Signalling and behaviour of *Globodera pallida* in the rhizosphere of the trap crop *Solanum sisymbriifolium*

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DOCTOR OF PHILOSOPHY

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Signalling and behaviour of *Globodera pallida* in the rhizosphere of the trap crop *Solanum sisymbriifolium*

by

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Signalling and behaviour of *Globodera pallida* in the rhizosphere of the trap crop *Solanum sisymbriifolium*

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Abstract

Potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida,* are economically important pests of potato (*Solanum tuberosum*) crops in potato growing regions worldwide. Integrated management is under threat, with effective nematicides increasingly being withdrawn on environmental and health grounds. Alternative strategies are urgently needed and trap cropping could be one of them.

The non-tuber-bearing *Solanum sisymbriifolium* is regarded as an effective trap crop for PCN with strong hatching ability and immunity to PCN infection and has been used in the UK and The Netherlands. However, its mode of action is unknown.

In order to shed light on the mode of action so that a novel control strategy could be identified, the interactions between *G. pallida* and *S. sisymbriifolium* were investigated using *in vitro* bioassays. In choice assays, *G. pallida* J2s were equally attracted to the roots of *S. sisymbriifolium* and to those of *S. tuberosum*. However, potato root diffusate (PRD), which is routinely used to induce PCN hatch, failed to attract *G. pallida* J2s in chemotaxis bioassays, indicating hatching factors (HFs) and soluble compounds present in PRD are not involved in attraction of *G. pallida* J2s to potato roots. The J2s invaded the roots of *S. sisymbriifolium* in large numbers but failed to develop further. To

facilitate continuous observation of nematode development, a novel *in vitro* method was devised with the use of Pluronic F-127, which requires no sterilisation, and the life cycle of *G. pallida* was successfully observed in *S. tuberosum* roots. Quantitative real-time polymerase chain reaction analyses of defence related genes of *S. tuberosum* and *S. sisymbriifolium* infected with *G. pallida* revealed up-regulation of the chitinase gene (*ChtC 2.1*) at 3 days post inoculation in *S. sisymbriifolium* but not in *S. tuberosum.* Electrospray ionisation-mass spectrometry analyses of root exudate extracts of the two *Solanum* species and subsequent bioassay-guided fractionation showed that the HF of *S. sisymbriifolium* differs from that of *S. tuberosum*. Previously, attention had been solely paid to the hatching ability of the root exudate of *S. sisymbriifolium*, but this study revealed for the first time that the aerial part extract possesses a significant hatching ability.

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Copies of publications

Signalling and behaviour of potato cyst nematode in the rhizosphere of the trap crop, *Solanum sisymbriifolium*

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Glossary (Definitions of terms)

1. Nematode behaviour to plants

Orientation

The process whereby animals establish or maintain their body attitude in relation to the external environment and can be divided to primary and secondary orientation. The former is regarding controlling the body attitude, for example, by orientation to the contact of surfaces, to dorsal light, to gravity, etc. Secondary orientation is superimposed on the primary orientation and is regarding the orientation to gradients of chemicals, humidity, potentials, etc. It is further divided into kineses and taxes (Fraenkel & Gunn, 1961).

Kinesis (kineses in pl.)

Movement that lacks directional orientation and depends on the intensity of stimulation (Lee, 2002).

Orthokinesis

Non-directional response in which an animal's speed of movement alters with changes in the intensity of stimulation (Lee, 2002).

Klinokinesis

Non–directional response in which an animal's rate of turning, or rate of change of direction, alters with changes in the intensity of stimulation (Lee, 2002).

Taxis (**Taxes** in pl.)

A type of behaviour concerned with the directed orientation of an animal in which it moves towards (positive) or away from (negative) the source of stimulation (Lee, 2002).

Klinotaxis

Movement along a concentration gradient by comparing intensities of stimulation by moving the anterior end from side to side (Lee, 2002).

Tropotaxis

Movement that involves the simultaneous comparison of the stimulation of two sense organs, for example, on the head and on the tail of a vermiform animal or on both sides of the body (Lee, 2002).

Random movement

In this study, random movement is defined as movement unrelated to influence of stimuli.

Attraction

In the narrowest sense, it is called attraction only if directed orientation reactions (klinotaxis or tropotaxis) participate in the finding of a source of stimulation. If undirected orientation reactions (orthokinesis or klinokinesis) participate, it is attraction in its wider sense (Klingler, 1965).

Attractant

Anything that draws is an attractant. More specifically, any stimulus which elicits a positive directive response may be termed an attractant (Dethier, 1947).

Long distant attractant (In nematology)

An attractant that draws a nematode to the root area (relevant to a scale of several cm) (Perry, 2005; Spence *et al.*, 2008).

Short-distant attractant (In nematology)

An attractant that draws a nematode to individual host roots (Perry, 2005; Spence *et al.*, 2008).

Local attractants (In nematology)

An Attractant that draws a nematode to preferred invasion sites (Perry, 2005; Spence *et al.*, 2008).

Repellent

Anything that repels is a repellent. Any stimulus that elicits an avoiding reaction may be termed a repellent (Dethier, 1947).

2. Plants to nematodes

Resistance

Describes the effects of host genes that restrict or prevent nematode multiplication in a host species (Trudgill, 1986).

Incompatible interaction

Between a parasite and a resistant host (McCarter, 2009).

Compatible interaction

Between a parasite and a susceptible host (McCarter, 2009).

Susceptibility

Vulnerable to invasion and reproduction by a specific nematode species or population (McCarter, 2009).

Tolerance

Independent of resistance (Cook, 1974; Paulson & Webster, 1972) and relates to the ability of a host genotype to withstand or recover from the damaging effects of nematode attack (Trudgill, 1991).

Immunity

Not subject to attack or infection by a specified pest or pathogen (ISF, 2005) (ISF: International Seed Federation).

3. Nematode

Diapause

“A physiological state of arrested growth and development, usually in response to unfavourable conditions, whereby development does not continue until

specific requirements have been satisfied, even if favourable conditions return" (Perry & Moens, 2006).

Quiescence

"A state of arrested development in response to unfavourable conditions, which is reversible when favourable conditions return" (Perry & Moens, 2006).

Virulence

1) "A measure of the ability of a nematode to cause damage, with a highly virulent isolate causing more damage than a weakly virulent isolate."
2) "A measure of the ability of a nematode to reproduce on a plant, especially when comparing populations of the same species for their ability to reproduce on resistant plants." (Perry & Moens, 2006).

Pathotype

"A group of individual nematodes with common gene(s) for (a) virulence and differing from gene or gene combinations found in other groups, which can be distinguished from others of the species by their pathogenicity on a specific host" (Perry & Moens, 2006).

Population

"A group of individuals of the same species at a given time and space" (Perry & Moens, 2006).

4. Others

Semiochemicals

Chemicals that cause inter- or intra-specific interactions between organisms (Huettel, 1986; Perry & Aumann, 1998).

Allelochemicals

Semiochemicals that cause a physiological or behavioural response between members of different species (Huettel, 1986; Perry & Aumann, 1998).

Pheromones

Semiochemicals that cause a physiological or behavioural response between members of the same species (Huettel, 1986; Perry & Aumann, 1998).

Rhizosphere

“The space around the root that is influenced by the root and its diffusates” (Perry & Moens, 2006).

Abbreviations

AChE	acetylcholinesterase
ca.	*circa*
*C*t	threshold cycle
dpi	days post inoculation
df	degrees of freedom
DW	distilled water
ESI-MS	electrospray ionisation-mass spectrometry
HF	hatching factor
HPLC	high-performance liquid chromatography
ISC	initial syncytial cell
J2	the second-stage juvenile (infective stage)
J3	the third-stage juvenile
J4	the fourth-stage juvenile
LSD	least significant difference
MS	Murashige & Skoog
NMR	nuclear magnetic resonance
PAL	phenylalanine ammonia-lyase
PCN	potato cyst nematode
PCR	polymerase chain reaction
PRD	potato root diffusate
qRT-PCR	quantitative real-time polymerase chain reaction
SED	standard error of the difference
SPE	solid phase extraction
TW	tap water
wpi	weeks post inoculation

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Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other university award without prior agreement of the Graduate Committee.

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Relevant scientific seminars and conferences were regularly attended at which work was often presented. Papers were prepared for publication: two published, with others under preparation.

Publications

1. SASAKI-CRAWLEY, A., CURTIS, R., BIRKETT, M., POWERS, S., PAPADOPOULOS, A., PICKETT, J., BLACKSHAW, R. & KERRY, B. (2010). Signalling and behaviour of potato cyst nematode in the rhizosphere of the trap crop, *Solanum sisymbriifolium*. *Aspects of Applied Biology,* 103**,** 45-51.

2. SASAKI-CRAWLEY, A., CURTIS, R., BIRKETT, M., PAPADOPOULOS, A., BLACKSHAW, R. & PICKETT, J. (2012). The use of Pluronic F-127 to study the development of the potato cyst nematode, *Globodera pallida*. *Nematology,* 14**,** 869-873.

Presentation and Conferences Attended:

1. Advances in Nematology (AAB meeting): Poster presentation
   15 December 2009, the Linnean Society of London

2. 3rd Symposium on Potato Cyst Nematode: Oral presentation
   14 – 15 September 2010, Harper Adams University College, Newport, UK

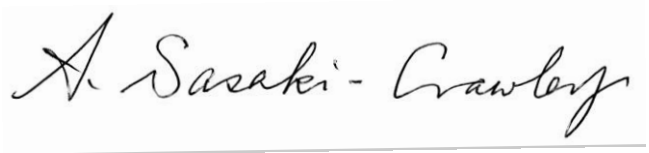
3. Joint 30th International European Society of Nematologists Symposium & 5th Phylloxera Symposium: Poster presentation
   19 – 23 September 2010, University of Natural Resources and Applied Life Sciences, Vienna

4. Advances in Nematology (AAB meeting): Oral presentation (Best student paper award)
   14 December 2010, the Linnean Society of London

5. The 43rd Organization of Nematologists of Tropical America Annual Meeting: Poster presentation
   4 – 8 September 2011, University of Coimbra, Portugal

6. Joint meeting of the Royal Entomological Society of London and the Soil Ecology Society: Oral presentation
   14- 16 September 2011, National Marine Aquarium, Plymouth, UK

7. Advances in Nematology (AAB meeting): Oral presentation (Best student paper award)
   13 December 2011, the Linnean Society of London



21 January 2013

Chapter 1

General Introduction

1.1. Nematodes

1.1.1. Plant parasitic nematodes

Plant-parasitic nematodes, all of which are obligate parasites, can devastate a wide range of crop plants, through the withdrawal of nutrients and the damage they inflict on host roots. Annual global agricultural losses are estimated to be > $100 billion (Sasser & Freckman, 1987; Koenning *et al.*, 1999; Chitwood, 2003a; Abad *et al.*, 2008) The groups of nematodes of the greatest economic importance are the sedentary endoparasites, with most of the damage being caused by the family *Heteroderidae*, which include *Globodera* spp. (potato cyst nematodes, PCN) and *Meloidogyne* spp. (root-knot nematodes, RKN) (Williamson & Gleason, 2003; Grunewald *et al.*, 2009).

1.1.2. Potato Cyst Nematodes (PCN)

Potato cyst nematodes (PCN), *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone, are regarded as the most damaging pests of the cultivated potato, *Solanum tuberosum* Linnaeus (Solanaceae), in the UK and mainland Europe (Fioretti *et al.*, 2002; Deliopoulos *et al.*, 2007). Within the European Union, the annual yield losses caused by PCN have been estimated at €300 million (Mulholland *et al.*, 1996; Haydock & Evans, 1998a). In the UK, approximately 9% of potato yield is lost annually owing to PCN (Evans & Stone, 1977), and the corresponding loss of market value has been estimated at £43 million, based on the mean value of the crop from 1990-1995 (Haydock & Evans, 1998a). Yield loss occurs with populations as low as 5 eggs per g of soil (Trudgill, 1986). In a recent survey of potato land, it was revealed that in

England and Wales, PCN were present in 64% of all sampled sites (Minnis *et al.*, 2002).

Both the potato and PCN originated in the Andes of South America, and both were introduced into Europe from the Andean region: the potato around 1570, but the nematode nearly 300 years later. It is believed that PCN were brought into Europe in the 1850s with potato collections from the Andes, in order to breed cultivars resistant to late blight (*Phytophthora infestans* (Mont.) de Bary) after the Irish potato famine in the late 1840s. Field damage caused by PCN was first observed in Germany in 1881, and they spread around the world largely through the trade in seed tubers of improved cultivars developed in Europe, to which soil infested with PCN was adhering (Evans & Brodie, 1980; Haydock & Evans, 1998a).

Initially, it was believed that there was only one species of PCN, *G. rostochiensis* (*Heterodera rostochiensis*), but a second species, *G. pallida* was recognised (Stone, 1972) through the introduction of *G. rostochiensis*-resistant cultivars, on which *G. pallida* could reproduce (Trudgill *et al.*, 1987). *Globodera rostochiensis* can be distinguished from *G. pallida* by the following differences: i) the morphology of the infective juveniles, *i.e.* a longer body length and stylet with *G. pallida*, ii) the colour of the adult females on the potato roots, *i.e.* yellow with *G. rostochiensis* and white with *G. pallida* (Turner & Rowe, 2006) (Figure 1.1). *Globodera rostochiensis* females turn from white through yellow to brown as they mature, whereas *G. pallida* females do not have the yellow phase (Vlachopoulos & Smith, 1993), hence they are called golden nematode and white/pale cyst nematode, respectively. Vlachopoulos and Smith (1993)

reported that the presence of aurone (a flavonoid) is responsible for the yellow/golden colour of *G. rostochiensis* females, which *G. pallida* females lack.

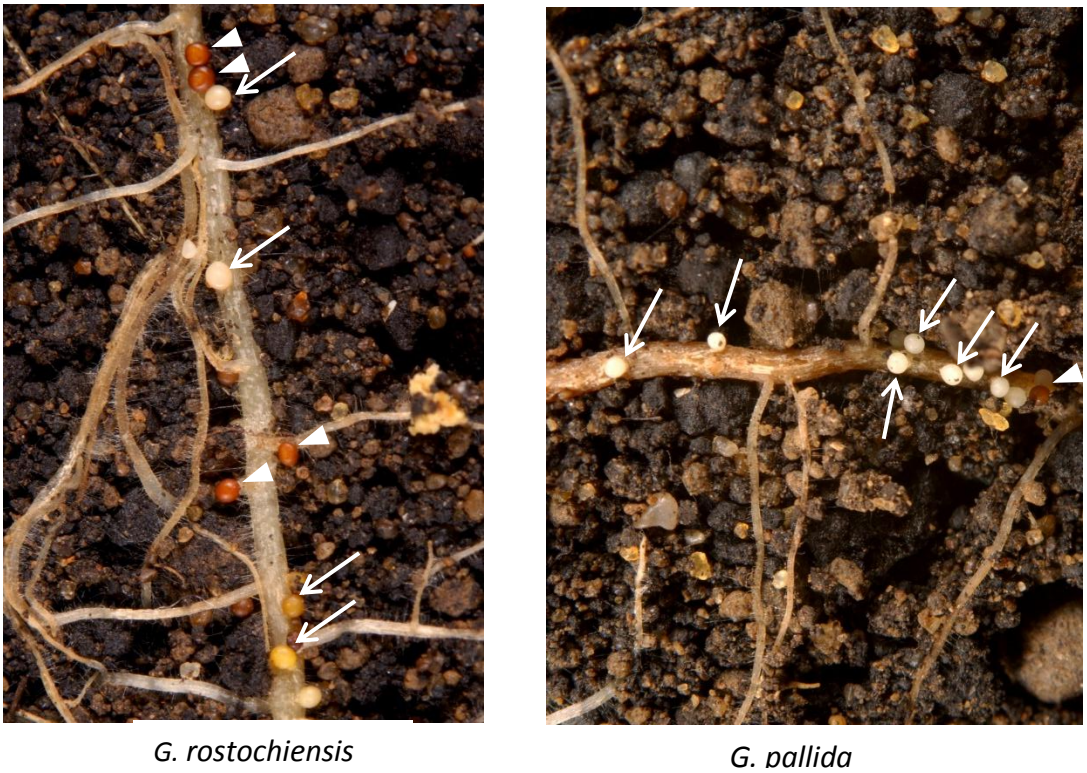


Figure 1.1. Adult females (*arrow*) and cysts (*arrowhead*) of *Globodera rostochiensis* and *G. pallida*. (From the Image Database of Rothamsted Research Visual Communications Unit).

New European Union (EU) Council PCN Directive

A new EU council Directive (2007/33/EC) came into effect on 1st July 2010, which i) recognises potato cyst nematodes (*G. rostochiensis* and *G. pallida*) as harmful organisms of potatoes, ii) involves official investigations to ensure no potato cyst nematodes are found in fields intended for seed potato production, and iii) determines the distribution of PCN in ware land (land for producing eating potatoes as opposed to seed potatoes), through harmonised

sampling, detection and identification methods across the EU. This new directive followed the recognition that there had been significant developments in the nomenclature and understanding of biology and epidemiology of PCN species and populations* and their distribution pattern since the adoption of the old Directive 69/465/EEC of 8 December 1969 (CEU, 2007; Hockland, 2010).

1.2. Biology of Potato Cyst Nematodes

1.2.1. Life cycle (Figure 1.2)

The infective second-stage juveniles (J2) (Figure 1.3a) of PCN, after invading roots near the root tip, migrate intra-cellularly through the cortical cells towards the vascular cylinder and induce a multinucleate feeding site called a "syncytium". A syncytium initiates with expansion of the initial syncytial cell (ISC) towards the vascular tissue by progressive cell-wall dissolution, resulting in the fusion of the protoplasts of neighbouring cells (Jones, 1981; Hussey, 1989; Golinowski *et al.*, 1997). The females of PCN, which mature after moulting through third (J3) and fourth (J4) stages, swell and burst through the epidermal layers of the root and become visible on the root surface. The adult males, which regain their vermiform shape, leave the root and fertilise the females. When the females die, their cuticles are tanned to form cysts (Figure 1.3b), which contain several hundred eggs (Trudgill *et al.*, 1996; Perry, 2002; Bridge & Starr, 2007). This tanning and hardening process to become cysts has been shown to be due to the activity of polyphenol oxidase by Ellenby (1946).

* See Glossary for definition.

Each of those eggs eventually contains a second-stage juvenile (J2). When the crop is harvested and roots decay, cysts are released from the roots and remain in the soil (Perry, 1978). Being protected by the cyst, the following generation of J2 in eggs can survive in the soil for more than 20 years, through the combination of diapause* and quiescence* until a suitable host is found nearby (Wright & Perry, 2006; Turner & Rowe, 2006). The hatching of the J2 from eggs and subsequent emergence from the cysts occurs in response to root exudates from their host plants, which enables PCN to synchronise their life cycle with the presence of their host plants in the soil (Clarke & Perry, 1977; Sharma & Sharma, 1998; Perry, 2002).

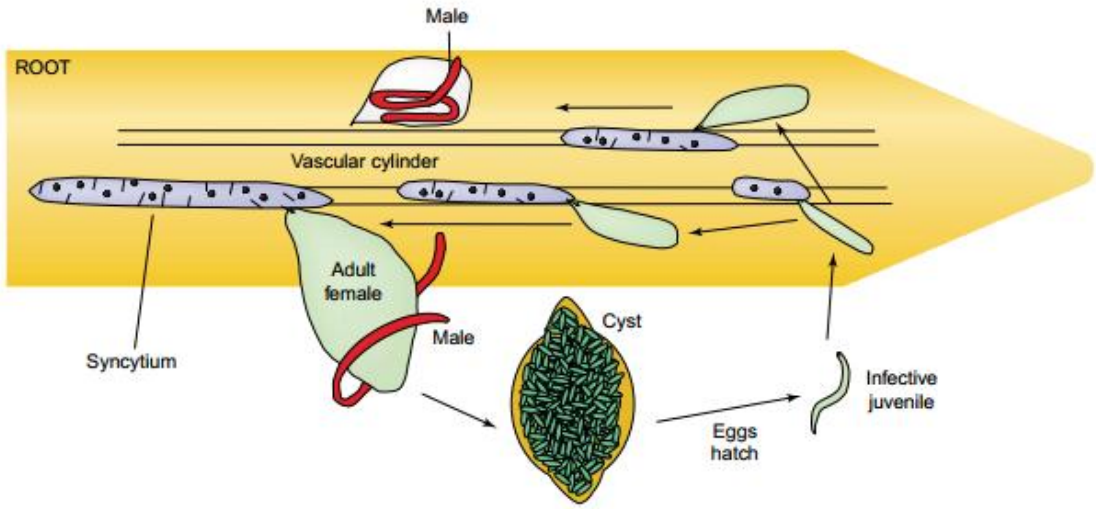
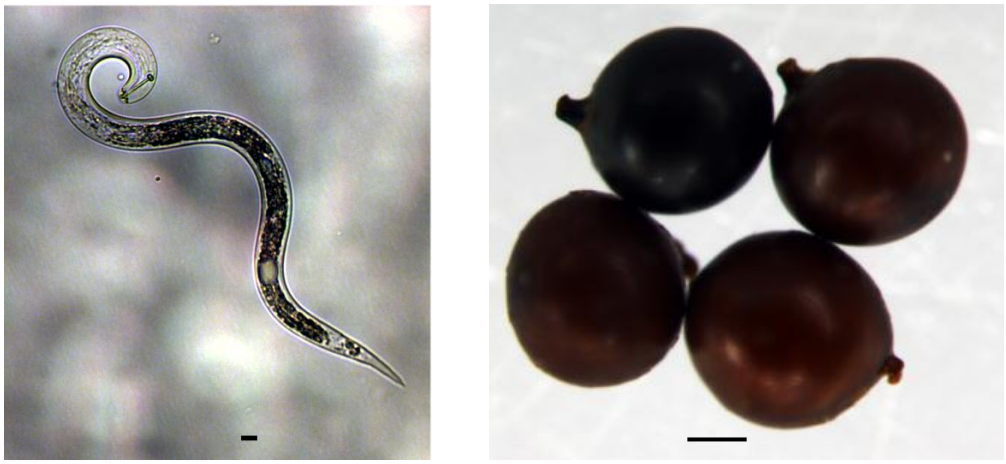


Figure 1.2. Life cycle of cyst nematodes (Williamson, V. M. & Gleason, C. A. (2003). Plant-nematode interactions. *Current Opinion in Plant Biology,* 6, 327-333). Reprinted with permission of Elsevier.

* See Glossary for definition.



(a) (b)

Figure 1.3. *Globodera pallida*. (a) Second-stage juvenile (J2). Bar = 10 µm, (b) cysts. Bar = 250 µm.

1.2.2. Hatching of J2

A small proportion of PCN J2s hatch spontaneously in the absence of a host crop each spring, but the majority require hatching stimulation in root exudates from the host plants in the spring (Trudgill *et al.*, 1996; Devine *et al.*, 1999) to terminate quiescence and to trigger their hatching (Jones *et al.*, 1998). This almost total dependence on host root diffusates for hatching reflects their restricted host range, which is mainly the Solanaceae family, such as potato, tomato and aubergine (Turner & Rowe, 2006). This is a stark contrast to other sedentary endoparasitic nematodes with a wide host range, such as *Heterodera schachtii* Schmidt (beet cyst nematode) and root-knot nematodes (*Meloidogyne* spp.), whose hatching does not depend on host root diffusates, although root diffusates enhance the rate of hatch (Perry, 1997; Karssen & Moens, 2006).

The egg inside the PCN cyst contains a coiled J2 surrounded by the perivitelline fluid. Trehalose in this perivitelline fluid causes an osmotic stress on the unhatched J2: thus, the water content in the J2 is lower than the normal state (Perry, 2002), which is estimated to be 67.3% in *G. rostochiensis* (Ellenby & Perry, 1976). This partial dehydration inhibits the J2's movement, which enhances the J2's survival, as energy reserves can be maintained (Perry, 2002). In order to activate the J2 to hatch, the osmotic pressure has to be removed (Wright & Perry, 2006). After the exposure to hatch stimulation from a hatching factor (HF) in host root exudates, permeability changes in the inner lipid layer of eggshell membranes mediated by calcium ions (Ca^{2+}): Ca^{2+} is understood to be removed from sites in the lipoprotein layer by HFs (Clarke & Hennessy, 1983; Clarke & Perry, 1985). This permeability change in eggshell leads to the leakage of trehalose from the perivitelline fluid and a concurrent influx of water, which reduces the osmotic pressure in the perivitelline fluid: thus, the J2 becomes sufficiently hydrated to sustain continuous movement. The J2 explores the inner surface of the egg with its lips and stylet, followed by thrusting movements with the stylet. This causes perforations in the eggshell, which subsequently become a slit, through which the J2 hatches (Wright & Perry, 2006).

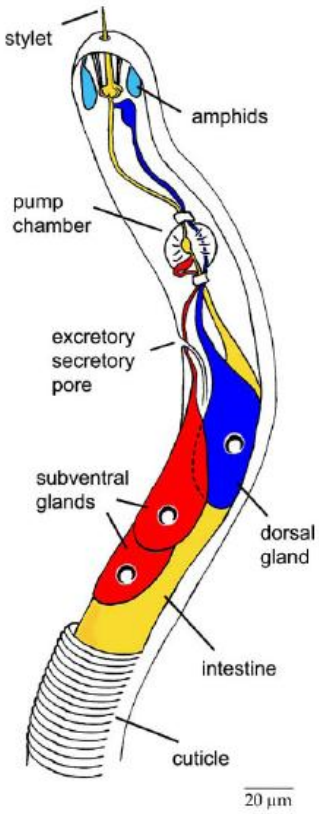
1.2.3. Orientation towards host plant roots

After hatching, PCN J2s move towards the roots of their host plant and invade them. However, this invasion has to happen swiftly, because the lipid reserves, which are the only energy source available until a syncytium is

induced, are utilised during movement (Storey, 1984). When lipid reserves fall below *circa* (*ca*.) 65% of the original level, motility and infectivity of the J2 is reduced significantly (Robinson *et al.*, 1987). Under optimal conditions for movement, the J2 of *G. rostochiensis* has an infective life of only six to 11 days after hatching (Robinson & Perry, 2006). Without feeding, the survival period of PCN J2s is less than 2 weeks (Robinson *et al.*, 1987), thus the ability of J2s to orientate towards host plant roots enhances their chance of survival (Curtis, 2008).

For the orientation towards the host plant, nematodes have to sense signals from the host, and chemo-orientation is essential for detection of host plant exudates. Nematodes detect their environment with sensory receptors, which mediate a variety of behavioural responses. The main sense organs are located anteriorly, and the amphids are the largest and most complex of the anterior sensory organs, which are situated on either side of the nematode mouth (Ward *et al.*, 1975; Lewis & Hodgkin, 1977; Riga, 2004) (Figure 1.4). The amphids are considered to be primarily chemosensory structures, although evidence from work on the free-living nematode, *Caenorhabditis elegans* Maupas, shows that they also have thermosensory and mechanosensory roles (Robinson & Perry, 2006). The phasmids, another paired sensilla, are located in the tails of many nematode species. Their chemosensory function is suggested by ultrastructural studies, but their precise role remains unclear (Jones, 2002) (Figure 1.4b).

(a)



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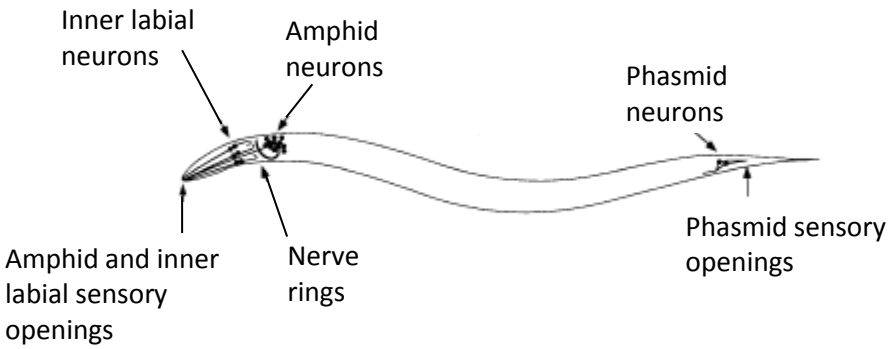


Figure 1.4. Amphids and phasmids. (a) Amphids in schematic representation of the anterior region of a J2 (sedentary endoparasitic nematode) (Vanholme, B., De Meutter, J., Tytgat, T., Van Montagu, M., Coomans, A. & Gheysen, G. (2004). Secretions of plant-parasitic nematodes: a molecular update. *Gene,* 332, 13-27), (b) amphid and phasmid neurons in a nematode (Hilliard, M. A., Bargmann, C. I. & Bazzicalupo, P. (2002). *C. elegans* responds to chemical repellents by integrating sensory inputs from the head and the tail. *Current Biology,* 12, 730-734). Both reprinted with permission of Elsevier.

1.3. Damage to crops by PCN

Aboveground damage to crops caused by PCN is not characteristic. Like most nematode-caused damage, infestation is observed in patches with stunting and chlorosis, which could be easily mistaken for symptoms that are results of water logging, soil compaction or malnutrition. With invasion by PCN J2s, roots cannot elongate sufficiently, and their architecture and function are seriously affected. As a result, PCN-infested plants are more likely to suffer from water and nutritional stress, hence poor growth and reduced yields (Blackshaw & Kerry, 2008).

1.4. Control of PCN

1.4.1. Resistant* cultivars

Potato cultivars, such as Maris Piper and Cara, with the *H1* gene which was discovered by Ellenby (1952), confer almost complete resistance to *G. rostochiensis*, but unfortunately not to *G. pallida* (Trudgill *et al.*, 1987). In the UK, earlier surveys estimated a lower incidence for *G. pallida* than *G. rostochiensis* (Brown, 1970). However, in recent years *G. pallida* has become more dominant in England and Wales, with *G. pallida* found in 92% of sites sampled, compared with only 33% with *G. rostochiensis,* which suggests that selection of *G. pallida* in field populations* has occurred by the overuse of the cultivars only resistant to *G. rostochiensis*, such as Maris Piper (Minnis *et al.*, 2002). For *G*. *pallida*,

* See Glossary for definition.

cultivars with only partial resistance are available, such as Vales Everest, Sante, Harmony and Innovator (Haydock, 2012), which reduce the multiplication rate of the nematode but rarely population levels (Trudgill *et al.*, 1987; Phillips & Blok, 2008). According to DNA studies, the pathotypes* of *G. pallida* are much more heterogeneous and variable than *G. rostochiensis,* which complicates the use of partially-resistant cultivars (Fleming & Powers, 1998). If overused, these partially resistant cultivars may lead to selection of virulent* populations (Haydock & Evans, 1998b). It was demonstrated by Turner (1990) and later Phillips and Blok (2008) that reproductive ability of *G. pallida* increased by selection pressure through continuous growth of partially resistant potato genotypes. Therefore, Phillips and Blok (2008) recommend that cultivars with different sources of resistance should be used, rather than one type being used repeatedly.

A resistance gene *Gpa2* confers resistance against a small set of field populations* of *G. pallida* pathotype Pa2 and has been cloned. Interestingly, *Gpa2* is highly similar in amino acid sequence to the *Rx* and *Rx2* genes, which confer resistance to Potato Virus X (van der Voort *et al.*, 1997; Sacco *et al.*, 2009). The limitation of *Gpa2* is, however, that it is resistant to only part of the field populations of one pathotype.

Although transgenic tomato lines carrying *Hero A* gene were highly resistant to all important European pathotypes of *G. rostochiensis* (Ro1, Ro3 and Ro5) and *G. pallida* (Pa1, Pa2/3 and Luffness), transgenic potato lines

* See Glossary for definition.

expressing *Hero A* gene failed to confer resistance to both *Globodera* species (Sobczak *et al.*, 2005). A similar result was reported with the *Mi* gene, which confers resistance against several root-knot nematode species, where the effort to transfer this resistance from tomato to tobacco was unsuccessful (Williamson, 1998).

1.4.2. Nematicides

The term “nematicide” is commonly used to include both the chemical compounds that directly kill nematodes (true “nematicides”) and those that paralyse the nematodes for a sufficient time to deplete their lipid reserves so that no injury is caused to plants (“nematistats” or “nematistatics”) (Haydock *et al.*, 2006). Nematicides can be divided to mainly two types according to mode of application: fumigants and non-fumigants (Schomaker & Been, 2006).

1.4.2.1. Fumigants

Fumigant nematicides are either compounds that are based on halogenated hydrocarbons or those that release methyl isothiocyanate. Compounds in the first category are exemplified by methyl bromide and 1,3-dichloropropene and believed to affect nematodes’ biochemical pathways in protein synthesis and respiration directly. The second category, compounds such as sodium *N*-methyldithiocarbamate (metam sodium), prevent respiration by affecting oxygen utilisation by nematodes with cyanide (Haydock *et al.*, 2006).

The production and consumption of one of the most effective and extensively used fumigants, methyl bromide, was phased out in January 2005 for developed countries, because it was recognised as an ozone-depleting substance under the Montreal Protocol, and control measures for methyl bromide were included in the Copenhagen Amendment in 1992 under the Montreal Protocol (UNEP, 2007) (UNEP: United Nations Environment Programme).

Telone II (1,3-dichloropropene) (Dow AgroSciences) was regarded as an important alternative. However, it has been suspended since March 2009, following the European Commission Decision of 20 September 2007 (2007/619/EC) concerning the non-inclusion of 1,3-dichloropropene in Annex I to Council Directive 91/414/EEC on the ground of the following concerns: potential contamination of groundwater, consumer exposure, risk to birds, mammals and aquatic organisms, and possible impact on non-target organisms (EFSA, 2009) (EFSA: European Food Safety Authority). Dow AgroSciences resubmitted the application for inclusion of 1,3-dichloropropene in Annex I to Council Directive 91/414/EEC in June 2008 and the final decision had been pending (EC, 2010) (EC: European Commission). However, the decision on non-inclusion was finally made on 20 Jan. 2011 (Dalli, 2011).

The remaining fumigant, metam sodium (sodium *N*-methyldithiocarbamate), was also not included in Annex I to Directive 91/414/EEC in 2009 and a period of grace will expire by 31 December 2014 (EC, 2012) (EC: European Commission). As metam sodium has been seen as an important alternative to Telone II, its phase-out would be a serious setback

for PCN control where the populations are too high for non-fumigants to manage, because non-fumigants only keep the nematodes away from the plant, whereas fumigants kill the eggs (reviewed in Certis (2008)).

Even by fumigants, however, research has revealed that PCN control achieved is limited to only 80 % even at best (Hockland *et al.*, 2000), and therefore treatment with non-fumigants has to follow (Certis, 2008).

1.4.2.2. Non-fumigants

Non-fumigant nematicides are represented by two main classes of chemical compounds, i) organophosphate such as ethoprophos and fenamiphos, and ii) carbamate such as oxamyl and aldicarb, which are applied to the soil as granular or liquid formations (Rich *et al.*, 2004; Haydock *et al.*, 2006). These act as acetylcholinesterase (AChE) inhibitors (Nordmeyer & Dickson, 1990; Opperman & Chang, 1990), and therefore prevent the nematode from locating the host plant root by disrupting chemoreception at low concentrations. At high concentrations, nematode hatch and movement is disrupted (Haydock *et al.*, 2006). Non-fumigant nematicides are an important part of PCN management, reducing root damage and nematode reproductive rates. However, potato tuber yield responses are variable (Trudgill, 1986) and repeated use can lead to reduced nematicide efficacy through the selection of soil microorganisms that are able to degrade them (Smelt *et al.*, 1987; Smelt & Leistra, 1992). Another important point is that *G. pallida* is less readily controlled than *G. rostochiensis* by non-fumigant nematicides, such as oxamyl. This is believed to be due to a combination of a slower and longer period of hatching of

G. pallida than *G. rostochiensis* and the short period of time persistence of the nematicides in the soil, which means *G. pallida* J2s are able to invade roots, after the nematicides have broken down to non-active concentrations in the soil (Whitehead, 1992). It has also been shown that utilisation of lipid reserves is much slower with *G. pallida* compared with *G. rostochiensis* (Robinson *et al.*, 1987). As the active ingredient of these non-fumigant nematicides is an AChE inhibitor (Nordmeyer & Dickson, 1990; Opperman & Chang, 1990), they are mostly nematistatic rather than nematicidal, thus the nematodes can recover once the chemical breaks down (Rich *et al.*, 2004), which means *G. pallida* J2s have a better chance to be able to recover and infect the host than *G. rostochiensis,* having greater lipid reserves left.

The effectiveness of non-fumigant granular nematicides depends on both placement within the soil and timing (Hockland *et al.*, 2000). For placement of non-fumigant granular nematicides in the soil, shallow incorporation at less than five cm brings no benefit for yield or PCN control and thus should be avoided. Instead medium-depth incorporation at no-deeper-than 20 cm should be aimed at (Woods & Haydock, 2000).

Like fumigant 1,3-dichloropropene, aldicarb, though being regarded as an effective granular nematicide without phytotoxicity, was also not included in Annex I to European Council Directive 91/414/EEC in 2003, with certain member states granted a period of grace until 31 December 2007 (CEU, 2003) (CEU: Council of the European Union). Whereas, oxamyl, another granular nematicide, was included in Annex I in 2005 (EC, 2006).

With their broad-spectrum activity, most nematicides (fumigants and non-fumigants) alter soil flora and fauna dramatically, which leads to a decrease of beneficial organisms (Chitwood, 2003b). There is mounting pressure from environmental lobbyists to reduce pesticide use (Stafford *et al.*, 2000).

1.4.3. Crop rotation

Crop rotation is an important component in managing PCN infestations, because the host range of PCN is narrow with only three cultivated host plants: potato, tomato and aubergine (Turner & Rowe, 2006). While other crops are grown, spontaneous hatching of J2 occurs, although on a small scale (Den Ouden, 1960; Greet, 1974), and hence, PCN populations decline (Trudgill *et al.*, 1996). This decline rate is estimated around 30 % a year (Cooper, 1953). With this figure, Hancock (1988) suggested that a break of seven to eight years from potato growing would be necessary to bring down the PCN level to that before the last potato crop. The NFU (National Farmers' Union, UK) protocol suggests that the minimum rotation length of one year in at least five, and preferably longer, is advisable (Haydock & Evans, 1998b). However, the current trend in the UK is that the number of potato growers has been reduced and potato production has become more specialised. Production has become more concentrated on a smaller area of land, thus with shorter rotations, which leads to population build-up of PCN, especially with *G. pallida* that has lower decline rates than *G. rostochiensis* (Minnis *et al.*, 2002). According to the studies by Greet (1974) and Den Nijs and Lock (1992), the rate of spontaneous hatching of *G. pallida* is much lower than that of *G. rostochiensis.*

Trudgill *et al*. (2003) argues that the effectiveness of crop rotation is determined by the length of the rotation and the annual rate at which the population declines, and warns that, with the decline rate slower than 30%, the increase of *G. pallida* is unlikely to be controlled by five to seven year rotations, even where nematicides are used.

1.4.4. Biological control

For PCN, nematophagous fungi are regarded as potential agents for biological control (Mankau, 1980). Two species in particular, *Pochonia chlamydosporia* (Goddard) Zare and *Paecilomyces lilacinus* (Thom) Samson, have been much studied and the latter species has been commercialised (Bioact®, Bioact Corporation Pty Ltd., Sydney, Australia) for use against cyst and root-knot nematodes (Kerry, 2000; Kiewnick, 2009). *Pochonia chlamydosporia* grows on the roots of plants and parasitises the females and cysts after they emerge from the plant roots (Kerry, 1980). The involvement of appressoria in the penetration of nematode eggs has been observed in both species (reviewed in Chen and Dickson (2004)). Tobin *et al*. (2008) reported the efficacy of *P. chlamydosporia* as a biological control agent against PCN in potato crops grown under commercial field conditions, and recommended it to be used as part of an integrated strategy with other control methods.

Recently, Mohan *et al*. (2012) reported that endospores of an isolate of Gram-positive bacterium *Pasteuria* sp. from pigeon pea cyst nematode, *Heterodera cajani* Koshy, adhered to the cuticle of *G. pallida* J2 and these

endospores completed their life cycle within *G. pallida* females. These *Pasteuria* sp.-infected females had no eggs and failed to tan, remaining creamy white.

Deliopoulos *et al.* (2008) reported that arbuscular mycorrhizal fungi (AMF) have potential to reduce *G. pallida* multiplication through a dual mechanism involving stimulation of nematode hatch and inhibition of root invasion, but emphasised that field experiments are needed to assess the feasibility of including AMF in *G. pallida* integrated management strategies.

1.4.5. Nematicidal substances from plants

Green manures of *Brassica juncea* (L.) Czern. & Coss. (Brassicaceae) lines appear to have nematicidal effects on *G. pallida* with high concentrations of 2-propenyl glucosinolate affecting egg viability (Lord *et al.*, 2011). However, just like broad-spectrum synthetic soil fumigants, non-target effects of such mustard fumigation ought to be assessed carefully. The impact of mustard fumigation on beneficial entomopathogenic nematodes that control pests such as Colorado potato beetle (*Leptinotarsa decemlineata* Say) and root-knot nematodes has been reported (Ramirez *et al.*, 2009; Henderson *et al.*, 2009), although these pests are not established in the UK.

1.4.6. Novel control strategies

There is a potential for cystatins - plant cysteine proteinase inhibitors - to control nematodes through impairing protein digestion (reviewed in Atkinson *et al.* (1998)). Lilley *et al.* (2004) reported that one of the potato lines expressing

cystatin at feeding sites achieved 70 ± 4% resistance to PCN and a yield of 98 ± 5% relative to values for the untransformed *S. tuberosum* L. cv. Desiree.

In view of minimising adverse effects on environment and human health, highly targeted disruption of neurotransmitter and neuromodulator signalling of nematodes may be exploitable as a control strategy. For example, the importance of neuropeptides, especially FLPs (FMRFamide (Phe-Met-Arg-Phe-NH_2)-like peptides), in physiological systems of nematodes, such as feeding, locomotion and reproduction, is reviewed by Holden-Dye and Walker (2011). Interestingly, Kimber *et al.* (2007) reported that *flp* genes in *G. pallida* J2 are susceptible to RNA interference (RNAi) and therefore silencing of *flp* genes through RNAi may be a novel control strategy, although the authors admitted that translation of *in vitro* observations to *in vivo* control measures is a challenge. Important physiological roles of neuropeptides have also been identified in *C. elegans* (Papaioannou *et al.*, 2005; 2008a; 2008b).

Liu *et al.* (2005) reported efficacy of transgenic potato plants expressing synthetic chemoreception-disruptive peptides at low concentrations in suppressing development of *G. pallida*: one that inhibits AChE and other that binds to nematode nicotinic receptors in cholinergic neurones. These synthetic peptides are mimics of nematicides - aldicarb and levamisole respectively - and the authors suggest there is a benefit of delivering two different peptides in a stacked defence so that any potential resistance breaking would be delayed. However, the suppression of female development was quite limited, with 61 ± 4 % at best. Green *et al.* (2012) reported effectiveness of transgenic cv. Desiree potato lines secreting a synthetic chemodisruptive peptide (disulphide-

constrained peptide), which inhibits chemoreception of cyst nematodes by binding to nematode acetylcholine receptors, in both containment and field trials. The resistance level achieved was up to 77 ± 4 % relative to untransformed cv. Desiree. With the peptide expressed at the root tip, roots seem to be protected from nematode invasion. Green *et al.* (2012) therefore suggested that by combining this with an anti-feedant trait of a cystatin (Lilley *et al.*, 2004), an additive effect of resistance against *G. pallida* could be expected. The authors also confirmed that unlike nematicides there was no impact on the non-target nematode soil community by either the plants expressing the peptide or those expressing a cystatin in nematode feeding cells.

1.4.7. Integrated control

As each control measure has its limitations and drawbacks, integrated control strategies with partially resistant cultivars against *G. pallida* should be applied to infested land (Trudgill *et al.*, 1987; Trudgill *et al.*, 2003). However, even with the use of granular nematicides, if the rotation is too short, it often increases the number of nematodes (Haydock & Evans, 1998a). Therefore, rotations should be made longer, and alternative control measures, such as trap cropping, need to be incorporated (Trudgill *et al.*, 2003).

1.4.8. Trap cropping

The concept of trap cropping is to stimulate hatch of PCN J2s from eggs by root exudates, but the reproduction of the next generation is prevented, either by the destruction of the trap crop or the use of a trap crop that is

resistant or immune* to PCN infection, such that soil infestations decline (Scholte, 2000a). Scholte (2000b) reported the success of growing potato as a trap crop in spring for the control of PCN, even when the trap crop was alternated with a susceptible potato cultivar as a main crop. Soil infestation was reduced by 96% on average compared with the fallow control. However, the author acknowledged that the potato is not an ideal trap crop, because: i) the timing for destroying the crop is crucial to avoid the development of adult females and ii) it is necessary to destroy the progeny tubers with a systemic herbicide in order to avoid volunteers in the following year, which unless removed will enable the nematode population to increase. Scholte (2000b) also discovered that using potato as a trap crop in soil infested with root-knot nematodes, mainly *Meloidogyne hapla* Chitwood, led to an increase of *M. hapla* population in the soil compared with fallow. Potato is a good host for several species of root-knot nematodes, with *Meloidogyne chitwoodi* Golden, O'bannon, Santo & Finley and *M. hapla*, being most common in temperate climates (Bridge & Starr, 2007). If the field contains root-knot nematodes, the use of potato as trap crop may bring serious consequences, although root-knot nematodes are not established in the UK.

For these reasons, Scholte (2000c) decided to search for a non-tuber-bearing trap crop with resistance to PCN, a high hatching effect and good plant performance under temperate conditions. Ninety accessions of non-tuber bearing Solanaceae were screened, as it is known that hatching factors for PCN are only produced by species of the Solanaceae (Scholte, 2000c). The findings

* See Glossary for definition.

revealed that only *Solanum sisymbriifolium* Lamarck (Solanaceae) (Figure 1.5) combined strong hatch stimulation with full resistance to both *G. rostochiensis* and *G. pallida* and good plant performance under Dutch environmental conditions. Scholte and Vos (2000) confirmed that it grows well under temperate climatic conditions. Timmermans (2005) reported that after 150 days of growth, the PCN population density was reduced by 75% on average.

Figure 1.5. *Solanum sisymbriifolium.*

Solanum sisymbriifolium

Solanum sisymbriifolium Lamarck (Solanaceae), commonly known as wild tomato or sticky nightshade, is a shrubby weed originating from South America (Becker & Frieiro-Costa, 1988). It has divided leaves, which resemble those widely seen in the genus "*Sisymbrium"* of the family Brassicaceae, hence it is so named (Knapp, 2012). It is used for traditional medicine, *e.g.* roots are used as antihypertensives in Paraguay (González Torres, 1992), and as diuretic, analgesic, antisyphilitic and hepatoprotectives in Argentina (Filipov, 1994). The berries and leaves contain a glycoalkaloid, solasodine, which is used as an active constituent of oral contraceptives (Pandeya *et al.*, 1981).

Solanum sisymbriifolium has become an invasive weed in South Africa since the introduction at the end of the 19th century, and in some areas good quality pasture lands and forestry firebreaks have been invaded (Du Toit & van der Merwe, 1941; Nel, 1988; Zimmermann *et al.*, 2004).

Solanum sisymbriifolium as a trap crop

According to Scholte and Vos (2000), *S. sisymbriifolium* develops an extensive root system that penetrates to deeper soil layers than potato, with potential to affect more PCN cysts. Timmermans *et al*. (2006) pointed out that the advantages of *S. sisymbriifolium* are not only that the ecological balance in the soil is not disturbed, but also that its roots colonise the soil to a greater depth, which is never reached by fumigant application. Unlike potato, *S. sisymbriifolium* is also highly resistant to root-knot nematodes, such as *Meloidogyne hapla* and *M. chitwoodi* (Scholte & Vos, 2000), which are parasitic

on potato in temperate climates (Bridge & Starr, 2007). However, a recent study by Dias *et al.* (2012) reported *S. sisymbriifolium* was susceptible to *M. hapla,* although resistant to *M. chitwoodi*. In countries like Portugal, *Meloidogyne* spp. such as *M. javanica* (Treub) Chitwood, which attacks potato in tropical climates (Bridge & Starr, 2007) and *M. chitwoodi* appear to coexist with PCN (da Conceicao *et al.*, 2009) unlike in the UK, and therefore, careful monitoring is necessary. *Solanum sisymbriifolium* is reported to be highly resistant against several isolates of late blight (*Phytophthora infestans*) with low infection efficiency and lesion growth rates (Timmermans, 2005).

For the incorporation of *S. sisymbriifolium* as a trap crop into crop rotations, Timmermans *et al*. (2007) suggested that it should be included as an extra crop into the rotation in such a way that a crop with commercial value would not have to be sacrificed. The results of their experiments indicated that the part of the season when *S. sisymbriifolium* can be grown successfully is restricted and that the optimum planting season in The Netherlands is between early May and the end of July, as the base temperature for emergence (germination) of this plant is estimated around 9°C. In the UK, it is advised not to sow the seeds (the brand name “Foil-sis” by Branston Ltd) before the soil temperature reaches *ca.* 12°C, and therefore usually the sowing timing is from May to mid-July. Recommended seeding rates of *S. sisymbriifolium* are 30-50 plants/m^2 in order to be effective, and weed control by chemicals, such as bentazone, is also required as *S. sisymbriifolium* is slow to establish. In the field, germination normally occurs 12 to 14 days after sowing, but this may be very patchy and the growth can be slow in the first four to six weeks, although the

plants do become vigorous once established. In order to ensure *S. sisymbriifolium* would not become weed, it is recommended to top the crop before berries fully form, which also encourages regrowth. After growth, *S. sisymbriifolium* is chopped up and ploughed into the soil as green manure (personal communication by D. Nelson, Branston Ltd.).

As for the length of growth in the field to achieve the maximum hatching effect on PCN, Timmermans *et al*. (2006) reported that the hatching percentages of *G. pallida* by *S. sisymbriifolium* increased with the longer duration of crop growth: from 47% after 6 weeks to 75% after 21 weeks of crop growth. In the UK, 10 to 14 weeks of growth is recommended to be cost effective, although it can be left longer. In 2011, *S. sisymbriifolium* was planted in approximately 300 ha in the UK (personal communication by D. Nelson, Branston Ltd.). As *S. sisymbriifolium* induces hatch of PCN, it can be inferred that its roots exude similar hatching factors (HFs) to those of potato, *S. tuberosum.* For potato, the presence of at least 10 HFs active towards *G. rostochiensis* has been reported (Devine *et al.*, 1996; Devine & Jones, 2000a). Purification and characterisation studies of HFs have been difficult, because HFs are present in only trace amounts in potato root exudates. So far, only one HF has been identified, named solanoeclepin A, with the empirical formula $C_{27}H_{30}O_9$ (Mulder *et al.*, 1996), and with a characterised structure (Schenk *et al.*, 1999), (Figure 1.6). The question is whether the roots of *S. sisymbriifolium* also exude solanoeclepin A to elicit hatch of PCN J2s.

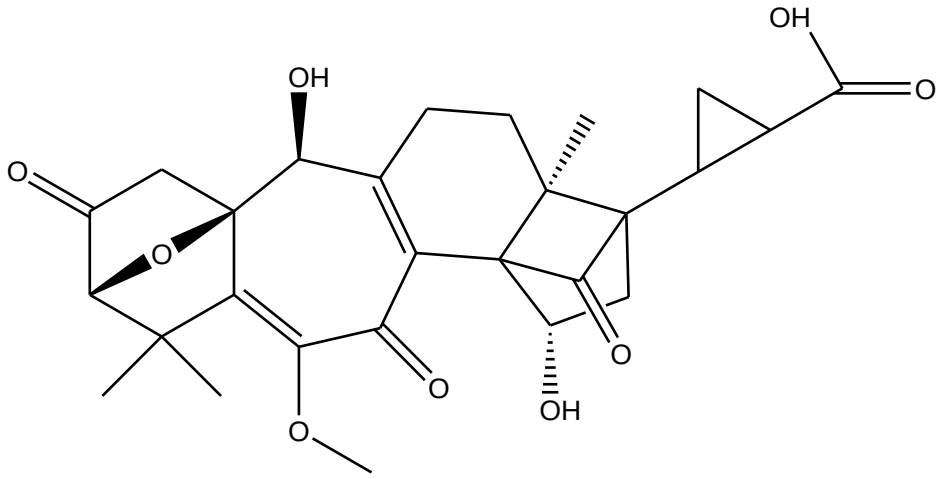


Figure 1.6. Solanoeclepin A ($C_{27}H_{30}O_9$).

1.5. Aim, objectives and hypotheses

The overall aim of this project is to identify potential targets for chemical or genetic intervention to control PCN by investigating the mode of action of *S. sisymbriifolium,* which induces hatch of PCN J2s and yet is immune* to the infection with the nematodes.

The project is focused on *G. pallida*, because it is the dominant species in England and Wales now, and is more difficult to control than *G. rostochiensis* as explained in section 1.4 above.

The hypotheses for this project were:

1. The signalling and behaviour of *G. pallida* in the rhizosphere of *S. sisymbriifolium* is different from the host plant *S. tuberosum* L. cv.

* See Glossary for definition.

Desiree, and therefore *G. pallida* J2s are neither attracted* to nor invade and develop in *S. sisymbriifolium* roots.

2. The continuous developmental study of *G. pallida* J2s can be successfully conducted in Pluronic F-127 aqueous solution.
3. Defence-related genes are up-regulated in the roots at an early stage of infection with *G. pallida* in *S. sisymbriifolium* but not in *S. tuberosum* Desiree, which might contribute to the immunity of *S. sisymbriifolium* against *G. pallida*.
4. A potato-derived HF, solanoeclepin A, is exuded from the roots of *S. sisymbriifolium*.

The specific objectives of this project were:

1. To investigate comparatively the behaviour of *G. pallida* J2s towards the roots of *S. sisymbriifolium* and *S. tuberosum* L. cv. Desiree (Chapter 3).
    a. To investigate whether *G. pallida* J2s are attracted to the roots of *S. sisymbriifolium*.
    b. To investigate whether *G. pallida* J2s invade the roots of *S. sisymbriifolium*.
    c. To establish the difference in development of *G. pallida* J2s in the roots of *S. sisymbriifolium.*
2. To investigate whether Pluronic F-127 aqueous solution is an ideal medium for *in vitro* development bioassays (Chapter 4).

* See Glossary for definition.

3. To compare the defence responses induced by *G. pallida* infection in *S. tuberosum* Desiree and *S. sisymbriifolium* (Chapter 5).
4. To investigate whether the roots of *S. sisymbriifolium* also exude solanoeclepin A, a HF of *S. tuberosum* (Chapter 6).

Chapter 2

General Materials and Methods

2.1. Nematodes (*Globodera pallida*)

The nematode population used was cultured on *S. tuberosum* L. cv. Maris Piper in a soil collected from a *G. pallida*-infested field in Waddington, Lincolnshire, UK. In autumn, after the aerial parts died back, the tubers and the whole plants were lifted and the soil was collected in soil bags and placed in a drying cupboard. When dry, the soil was washed through a Fenwick can to extract cysts (Fenwick, 1940). The cysts were separated from debris and stored at 4^{o}C.

In order to obtain the J2s, the cysts were first soaked in tap water for three to five days to hydrate, and then water was replaced with potato root diffusate (PRD) diluted four times with tap water and left in the dark at *ca*. 18 °C (Clarke & Perry, 1977). Hatched J2s were collected every 24 to 48 h, cooled to 5 °C for 30 min before being centrifuged at 2500 rpm for five minutes and used immediately.

Potato root diffusate (PRD) was collected from a four to eight-week-old *S. tuberosum* L. cv. Desiree grown from a piece of tuber with a sprout in a 15 cm-diameter pot with weed mix (80 % sterilised loam, 20 % lime free grit (3-6 mm), 2 kg/m^3 Osmocote) with sufficient water given to collect *ca.* 100 ml of run-off. The run-off was subjected to gravity filtration (using a Whatman No.1 filter paper) and stored in aliquots at -20 °C.

2.2. Plant materials

2.2.1. *Solanum sisymbriifolium*

Seedlings were grown from seeds (Branston Ltd., UK) sown in a small multi-cell seed tray with weed mix for germination at 20 °C (day) / 20 °C (night) with 16:8 hours of light:dark.

2.2.2. *Solanum tuberosum* L. cv. Desiree

Plantlets were grown at *ca.* 20 °C from small sprouts removed from tubers by soaking them in distilled water.

2.2.3. *Triticum aestivum* L. cv. Paragon (a spring wheat)

Seedlings were grown on a distilled water-moistened Whatman No. 1 filter paper in a Petri dish at *ca.* 20 °C.

2.3. Assumptions of ANOVA and Statistical system used

After each ANOVA, residual plots were checked to ensure that the assumptions of the analysis (Normal distribution, constant variance and additivity of treatment effects) had not been violated.

The statistical system used was GenStat® (2011) Fourteenth Edition, © Lawes Agricultural Trust (Rothamsted Research), VSN International Ltd., UK.

Chapter 3

Behaviour of *Globodera pallida* J2s towards roots and root exudates of *Solanum tuberosum* L. cv. Desiree and *S. sisymbriifolium*

Introduction

Although it is known that *S. sisymbriifolium* induces strong hatching of *G. pallida* J2s and yet does not allow the completion of their life cycle, their behaviour prior to root invasion and an early stage (up to three weeks) of infection has not been reported. Roberts & Stone (1983) started the observation of the invasion by *G. pallida* J2s at 3 weeks post inoculation (wpi) as opposed to 1 wpi with other *Globodera* species, with a susceptible plant species *Solanum prinophyllum* Dunal (Solanaceae) as a positive control. Scholte & Vos (2000) and Scholte (2000a; 2000c) studied the abilities of *S. sisymbriifolium* to induce hatching and to prevent reproduction of PCN, but did not examine the development of nematodes within roots.

Experiments were set up to test the hypothesis that "the signalling and behaviour of *G. pallida* in the rhizosphere of *S. sisymbriifolium* is different from that of the host plant *S. tuberosum* L. cv. Desiree, and therefore *G. pallida* J2s i) are not attracted*, ii) do not invade, iii) do not develop in *S. sisymbriifolium* roots."

Regarding hypothesis i), the generally supported theory is that host roots are attractive to plant-parasitic nematodes (Prot, 1980). However, clarification of the concept of attraction is necessary. Dethier (1947) defined that "anything that draws is an attractant* and more specifically any stimulus which elicits a positive directive response may be termed an attractant". Based on this definition, Klingler (1965) defined attraction as follows: in the narrowest sense it is called

* See Glossary for definition.

"attraction" only if directed orientation reactions (taxis*) participate in the finding of a source of stimulation; if undirected orientation reactions (kinesis*) participate, it is attraction in its wider sense. When a chemical causes an insect to slow down or stop its locomotion (orthokinesis*) or to change the rate of turning (klinokinesis*), the chemical is termed an "arrestant" (Kennedy, 1978). If nematodes are found around roots, it may be the result of their reactions to arrestants or attractants. And therefore, the current study redefined Klingler (1965)'s definition of "attraction in the wider sense" as "arrestment/attraction", which may involve kinesis (undirected) but does not exclude taxis (directed), and "attraction in the narrowest sense" as "attraction", which involves only taxis (directed).

Both "arrestment/attraction" and "attraction" responses of *G. pallida* J2s to the roots of *S. sisymbriifolium* were tested in two different set-ups. For the assessment of "arrestment/attraction", the movement of *G. pallida* J2s in response to the roots of *S. sisymbriifolium* was compared with that to *S. tuberosum* L. cv. Desiree (host, positive control) and also with a known non-host plant, *Triticum aestivum* L., cv. Paragon (spring wheat, negative control) in separate dishes. For the assessment of "attraction", two-way choice assays were conducted to examine whether J2s would have any preference for any of the plants tested in the following combinations: between *S. tuberosum* Desiree (host) and *S. sisymbriifolium* (trap crop), *S. tuberosum* Desiree (host) and wheat

* See Glossary for definition.

(non-host), *S. sisymbriifolium* (trap crop) and wheat (non-host) and *S. tuberosum* Desiree (host) and no plant.

Nematodes move towards the roots of their host plants, presumably responding to allelochemicals* exuded by the plant (Perry & Aumann, 1998). A further question asked was: will *G. pallida* J2s respond to potato root diffusate (PRD) as they do to a living plant? Electrophysiological analysis showed that *G. rostochiensis* J2 responded to PRD, with electrical activity from the anterior end of the nematode being recorded (Rolfe *et al.*, 2000). This response could be interpreted as the evidence that PCN are attracted to PRD (taxis*, with directed orientation to PRD). On the other hand, it might be the evidence that PRD activates non-directional movement (kinesis*) when PCN are exposed to PRD. In order to clarify this question, chemotaxis and motility assays were conducted to test taxis and kinesis effects of PRD on *G. pallida* J2s.

For testing the hypothesis ii) and iii) described above, invasion and development bioassays were done in pot experiments to compare the extent of invasion by *G. pallida* J2s and their development, between the host, *S. tuberosum* L. cv. Desiree, and the trap crop, *S. sisymbriifolium*.

* See Glossary for definition.

Materials and methods

3.1. Movement of *G. pallida* towards the roots

3.1.1. Separate assays for individual plant species (arrestment/attraction assays)

3.1.1.1. Comparison between *S. tuberosum* L. cv. Desiree (potato, host) and *S. sisymbriifolium* (trap crop)

3.1.1.2. Comparison between *S. sisymbriifolium* (trap crop) and wheat (*Triticum aestivum*, non-host)

Nematodes

See section 2.1 of Chapter 2. General Materials and Methods.

Plant materials

See section 2.2 of Chapter 2. General Materials and Methods apart from the following:

Solanum sisymbriifolium

For the bioassay paired with *S. tuberosum* L. cv. Desiree (section 3.1.1.1.) only: seedlings were grown from seeds (Branston Ltd.) at *ca*. 25 °C on Gamborg's B5 Basal Medium with Minimal Organics (Sigma) with 0.15 % sucrose (Fisher Scientific), 0.8 % agar (Sigma) and Gamborg's Vitamin Solution 1000 × (Sigma), pH 6.4.

Assay

Pluronic F-127 (Sigma) was used, instead of agar, to carry out the assays, as it does not form a rigid gel, unlike agar, and so allows nematodes to move freely in three dimensions, that is, it can mimic soil conditions. Pluronic F-127 gels and liquefies over a narrow range of temperature (0.5°C), and the gelling temperature is related to its concentration. As the concentration increases from 18% to 50%, the gelling temperature decreases from 32°C to 11°C, which means that it liquefies as the temperature drops below the critical value for the particular concentration used (Gardener & Jones, 1984). On agar surfaces, nematodes typically lie on their sides, which may be unnatural (Robinson, 2004) and possibly may affect their behaviour. Lateral movement of the anterior end of a nematode within a three-dimensional matrix should permit orientation to concentration gradients by a nematode via comparing sensory input of the two amphids, whereas on the surface of agar the assay is two-dimensional and the comparison might not happen so easily, as the nematodes have to use head movements (up and down on the agar surface) to perceive signals (Robinson & Perry, 2006; Wang *et al.*, 2009a). At a concentration of 30% Pluronic F-127, Ko and Van Gundy (1988) reported that most J2s of *Meloidogyne incognita* were immobilised, because of its high viscosity. Wang *et al.* (2009b) used a concentration of 23% successfully to study the behaviour of *Meloidogyne* spp., and reported it was a semi-solid gel at room temperature but was liquid at and below 15°C. Ko and Van Gundy (1988) reported 20% had minimal effects on the test organisms in semi-solid state at room temperature

(22°C). The concentration of 20 to 23% with distilled water was employed for the current study. For further details of Pluronic F-127, see Chapter 4.

Circa 100 *G. pallida* J2s in a minimum volume of 0.1M phosphate buffered saline (PBS: sodium chloride (BDH), sodium dihydrogen phosphate (Fisher Scientific), disodium hydrogen phosphate (Fisher Scientific), pH 7.2) suspension were placed into a Petri dish (3.5 cm in diameter). As soon as the total number of the J2s was counted, 1 ml of Pluronic F-127 (Sigma) aqueous solution (20-23 w/v% with distilled water) was added and the J2s were randomly distributed in the gel. Four Petri dishes were prepared in this way for one experimental block, comprising one *S. sisymbriifolium* plantlet, one *S. tuberosum* Desiree or wheat plantlet, and their corresponding controls (see below). The area within 3 mm of the root surface was regarded as an *a priori* "arrestment/attraction zone". The zone was so called because the J2s were randomly distributed to begin with and the number of J2s found in the zone was recorded, not their directions. Therefore, J2s might have arrived in the zone as a result of a modification to their speed or rate of non-directional kinesis* reactions to allelochemicals from the root (arrestment), or directed taxis* reactions towards the root (attraction). Before the gel set, a plantlet (*ca.* 5 days old) was placed in the centre of a prepared Petri dish (Figure 3.1a), and the number of the J2s in the arrestment/attraction zone was counted immediately. This constituted an observation at time 0. For controls, no plantlet was used and, instead, the exact plantlet shape of the corresponding plant species was outlined on the lid of the Petri dish (Figure 3.1b), and the J2 counts made. The

* See Glossary for definition.

assay was therefore conducted using such pairs of Petri dishes. The reason to have such a control was to prove that the J2s did not arrive at the arrestment/attraction zone as a result of random* movement, which is defined in this study as movement unrelated to influence of stimuli. The time points for counting were at 30, 60 and 120 min after 0 min, and the number of J2s in the zone was recognised as those "arrested/attracted" by/to signals from the root. As Pluronic gel is a medium which allows the J2s to move in three dimensions, the J2s that are immediately under a root (or already inside the roots) cannot be observed. In order to overcome this problem, actual counting was carried out in the zone outside the "arrestment/attraction zone", and the number in the "arrestment/attraction zone" was determined by subtracting this count from the total number. This test was replicated five times for the assay on *S. sisymbriifolium* and *S. tuberosum* Desiree, and four times for the assay on *S. sisymbriifolium* and wheat. For these assays, a Vickers Instruments stereo dissecting microscope was used.

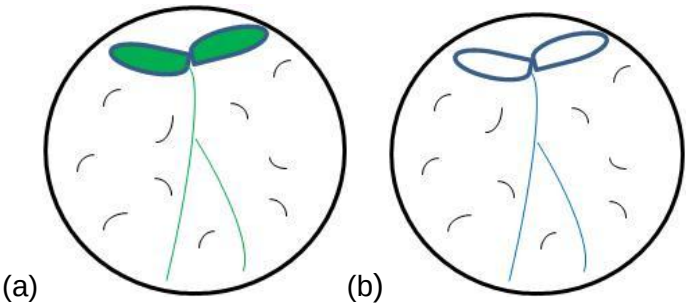


Figure 3.1. Illustrations of the arrestment/attraction assays. (a) A plantlet in Pluronic gel with *ca*. 100 *Globodera pallida* J2s, (b) its corresponding control: without the plantlet, only with its outline.

* See Glossary for definition.

Statistical analysis

The percentage of the J2s that were in the "arrestment/attraction zone" at the four time points were calculated for each of the dishes. Then, for each plant species, the ratio of plant-to-control at each time point was determined, in order to overcome the differences in the shape and the total length of roots between the plant species. For statistical analysis, split-plot in time ANOVA was implemented for five blocks (this being the replication) (four blocks for the assay between *S. sisymbriifolium* and wheat), using natural log of the calculated plant-to-control ratio. One block comprised four dishes: one for *S. sisymbriifolium*, one for potato/wheat, and their corresponding controls. Following ANOVA, relevant means were compared using the least significant difference (LSD) value at the (P = 0.05) level of significance, calculated from the standard error of the difference (SED) on the residual degrees of freedom (df) from the ANOVA. For details, see 3.1.1 of Appendix I: Statistical analysis.

3.1.2. Choice assays between two plant species (attraction assays)

3.1.2.1. Between *S. tuberosum* L. cv. Desiree (host) and *S. sisymbriifolium* (trap crop)

3.1.2.2. Between *S. tuberosum* L. cv. Desiree (host) and wheat (non-host)

3.1.2.3. Between *S. sisymbriifolium* (trap crop) and wheat (non-host)

3.1.2.4. Between *S. tuberosum* L. cv. Desiree (host) and "no plant"

Nematodes

See section 2.1 of Chapter 2. General Materials and Methods.

Plant materials

See section 2.2 of Chapter 2. General Materials and Methods.

Assay

A channel (2 x 8.5 cm) was cut out from a 3 % agar plate (*ca*. 30 ml, Sigma) in a Petri dish (9 cm in diameter) (Figure 3.2a), which was filled with 2 ml of 23 % Pluronic F-127. A plantlet (*ca.* 10 days old) to be tested was placed at opposite ends at 2 cm from the centre of the channel (Figure 3.2b). One hour later, a 5 mm-diameter filter paper (Whatman No.1) was placed at the centre of the channel, on which *ca.* 150 *G. pallida* J2s in 5 µl PBS suspension were added. The dish was placed inside a humidity chamber and incubated at 22°C in darkness. Migration pattern was quantified by counting the number of J2 that moved into four zones: 0.5-1, 1-1.5, 1.5-2 cm and over 2 cm in either direction from the centre at 30, 60, 90 and 120 min. The combinations of two-way choices tested were: 3.1.2.1. *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*, 3.1.2.2. *S. tuberosum* Desiree and wheat, 3.1.2.3. *S. sisymbriifolium* and wheat, and 3.1.2.4. *S. tuberosum* Desiree and “no plant”. For each combination of pairs of plants three plates were used.

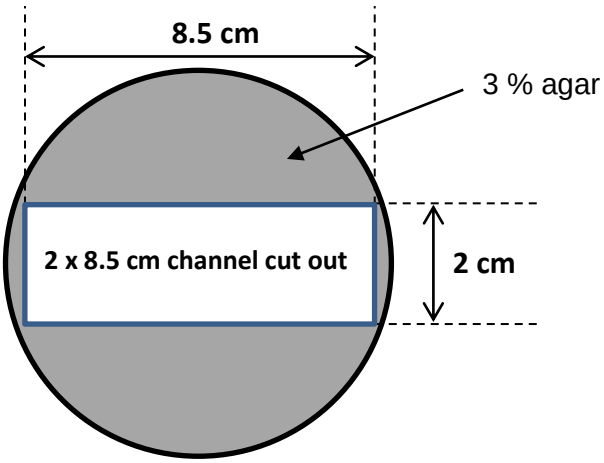
Statistical analysis

Counts of J2s were analysed by generalized linear model (GLM) assuming a Poisson distribution and using a log link function. The effects of plant species, time and distance were assessed, distance being nested within plant species given that any movement towards a particular plant species is

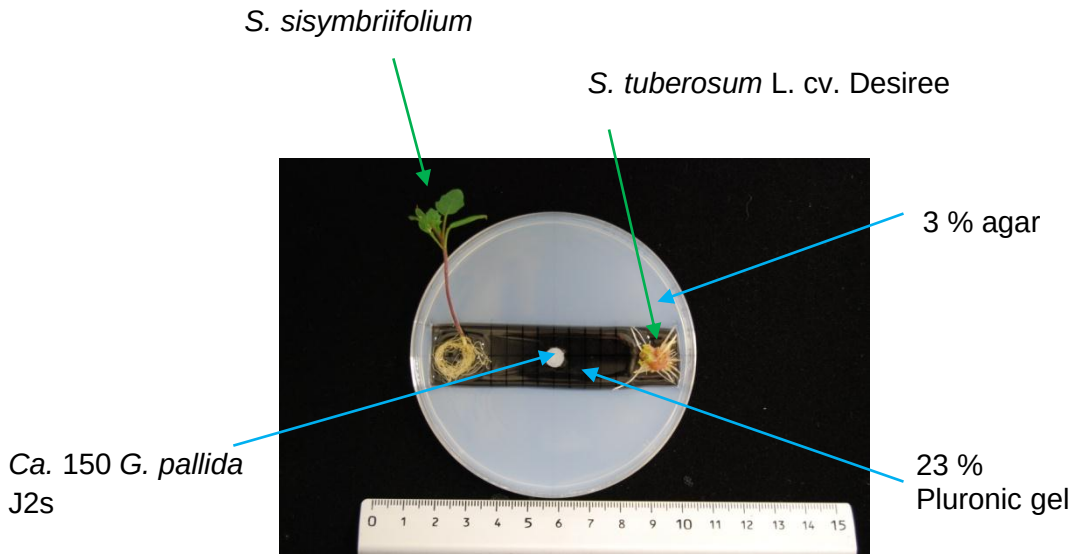
deemed as attraction towards that species. For example, for *S. sisymbriifolium* (*Ss*) and *S. tuberosum* (*St*), the model fitted was:

$$\text{Log}_e\,(count_{ij}) = Constant + PlantSpecies_i + \alpha\, Time + \beta_i\, Distance + \gamma_i\, Time + \delta_i\, Distance.Time + \varepsilon_{ij}$$

for *PlantSpecies* i = 1, 2 (1= *Ss*, 2 = *St*) and j = 1,…, 3 for the replicate dishes, where the *Constant* is the common underlying mean on the natural log scale, α is the overall coefficient of time, and β_i is the coefficient of distance, γ_i the coefficient of time and δ_i the coefficient of the interaction between distance and time for each plant species. Here, α, β_i and γ_i are rates of change per unit time or distance in log count of J2s, whereas δ_i are rates of change per unit time and distance which implies they are estimates of dispersion of the J2s. Also, ε_{ij} is the error term. All effects in the model were tested using analysis of deviance, involving approximate F-tests due to some overdispersion, *i.e*. extra variation than expected for a Poisson distribution. Despite this, the models fitted well to the count data as seen through the satisfactorily low residual mean deviances (see Appendix I). The results were shown as predicted back-transformed mean counts with standard errors (SE). For details, see 3.1.2 of Appendix I: Statistical analysis.



(a)



(b)

Figure 3.2. Two-way choice assays. (a) 3 % agar plate with a 2 x 8.5 cm channel cut out, (b) an example of a choice between *Solanum tuberosum* L. cv. Desiree and *S. sisymbriifolium*.

3.2. Chemotaxis of *G. pallida* towards root exudates of *S. tuberosum* L. cv. Desiree

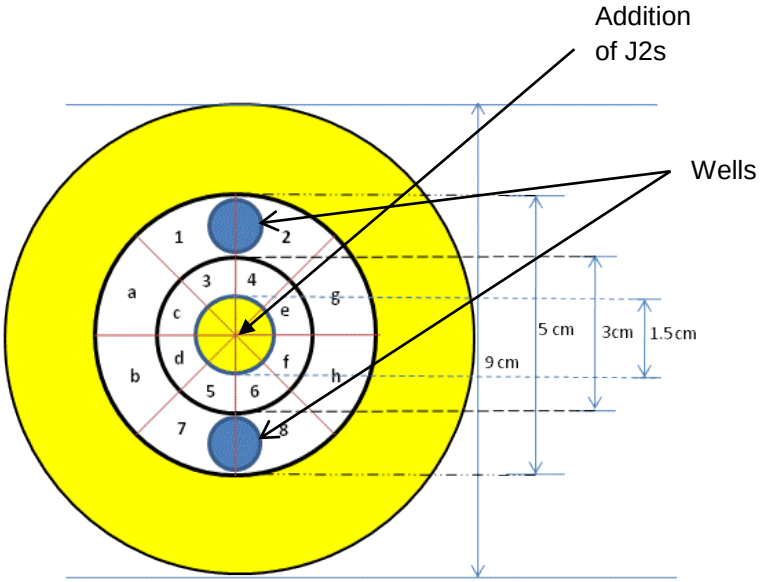
Assay

The chemotaxis assay, which tests directed orientation of J2s (taxis), was a modified version of the method by Wuyts *et al.* (2006). Nine cm-diameter Petri dishes were used, instead of 5 cm-diameter, in order to allow chemicals placed in the wells to diffuse more freely without being stopped by the edge of a dish, and a 1.5 cm-diameter circle in the centre was excluded from the segments (see Figure 3.3a) for scoring presence and absence of nematode tracks. Also, 0.75% Phytagel (Sigma) including 0.1% magnesium sulphate heptahydrate $MgSO_4.7H_2O$ (FSA Laboratory Supplies) was used, instead of water agar, so that nematode tracks could be observed under a microscope. Petri dishes were filled with 15 ml 0.75% Phytagel (including 0.1% $MgSO_4.7H_2O$). Wells of 1 cm diameter were made at opposite sides on the dishes at 2 cm from the centre, which were filled with 100 µl of chemical compounds to be tested: undiluted potato root diffusate (PRD: collected from a 6-week-old *S. tuberosum* L. cv. Desiree plant; for details, see section 2.1 of Chapter 2. General Materials and Methods), two-fold concentrated PRD, two-fold and four-fold diluted PRD with tap water, 1% acetic acid (AcOH, Fluka Chemie GmbH), 0.5 M calcium chloride ($CaCl_2$, Fisher Scientific) and distilled water (DW) / tap water (TW) as a negative control. One per cent AcOH and 0.5 M $CaCl_2$ were used as positive controls: a repellent and an attractant, respectively, following the study by Wuyts *et al.* (2006). Three replicate dishes for each chemical were used. The chemicals were left to diffuse for 3 h with lids

on the dishes, and then *ca.* 20 *G. pallida* J2s in a minimum volume of 0.1M PBS suspension were pipetted at the centre of each dish. It was decided that 3 h was enough for PRD in the well to diffuse to reach the inner most circle of 1.5 cm in diameter, having added a particle of methyl green (formula weight 458.5) to three-fold TW-diluted PRD. When the surface tension of the PBS suspension was lost, the dishes were covered with lids and incubated in the dark on a levelled platform at *ca.* 20 °C for 2 h. After 2 h, the presence or absence of nematode tracks was recorded for each segment: 16 in total with 8 attractant* (labelled with Arabic numerals, Figure 3.3a) and 8 repellent* (labelled alphabetically, Figure 3.3a) zones. For each segment, a score of 1 was given for presence of nematode tracks, and 0 for their absence. The chemotaxis factor (Cf) was calculated by dividing the sum of scores of the attractant zones by that of the repellent zones. A Cf greater than 2 meant attraction for the nematodes, while lower than 0.5 indicated repellence (Figure 3.3b).

* See Glossary for definition.

(a)



(b)

$$Cf = \frac{\text{attractant}}{\text{repellent}} = \frac{\text{Scores for } [1] + [2] + \ldots\ldots + [8]}{\text{Scores for } [a] + [b] + \ldots\ldots + [h]}$$

attractant	Cf > 2.0
repellent	Cf < 0.5
neutral	0.5 ≤ Cf ≤ 2.0

Figure 3.3. Chemotaxis assay. (a) 16 segments in a dish: 1 to 8 for attractant and a to h for repellent zones, with both the 1.5 cm-diameter circle in the centre and the outer ring in yellow excluded. For each segment, a score of 1 or 0 is given for the presence or the absence of nematode tracks, respectively, (b) an equation for calculating the chemotaxis factor (Cf), and standards for attractant/repellence assessment (Wuyts, N., Swennen, R. & De Waele, D. (2006). Effects of plant phenylpropanoid pathway products and selected terpenoids and alkaloids on the behaviour of the plant-parasitic nematodes *Radopholus similis, Pratylenchus penetrans* and *Meloidogyne incognita*. *Nematology,* 8, 89-101). Permission to reproduce these figures has been granted by the publisher, Brill.

Statistical analysis

One-way ANOVA was implemented for Cf, testing the significance of differences between treatments. Using the standard error of difference (SED) between the means for the treatments, least significant difference (LSD) at the 5% level was calculated for comparison of the treatments. For details, see 3.2 of Appendix I: Statistical analysis.

3.3. Motility assays with root exudates of *S. tuberosum* L. cv. Desiree

3.3.1. Experiment with PRD-hatched *G. pallida* J2s

Assay

In order to assess the effect of PRD on non-directional movement (kinesis) of *G. pallida* J2s, a modified version of the method by Dutta *et al*. (2012) was implemented in a 5.5 cm-diameter Petri dish with 4 ml of 0.75% Phytagel (including 0.1% $MgSO_4.7H_2O$). *Circa* 200 *G. pallida* J2s (hatched in PRD as described in section 2.1 of Chapter 2) in 2 µl PBS suspension were incubated with 15 µl of i) PRD (collected from a 7-week-old *S. tuberosum* L. cv. Desiree plant: 4-fold dilution with high purity distilled water: the same concentration as that routinely used for J2 hatching) and ii) high purity distilled water (control), in a 0.5 ml Eppendorf tube for 1 h in the dark at room temperature. At 1 h, 5 µl of each treatment was pipetted at the centre of the dish with three replicates. The number of J2s was counted in four circular areas of the Petri dish: the inner most circle where the J2s were added, area A (0.5 cm

from the innermost circle excluding this circle), area B (1.5 cm excluding the area A) and area C (2.5 cm excluding areas A and B) (Figure 3.4) at 30, 60 and 90 min after J2 introduction.

Statistical analysis

General ANOVA, taking account of dishes and areas within dishes as the design structure, was implemented for the percentage of the J2s in each area on a logit scale over three time points, testing the significance of differences between treatments (PRD and water), the effect of area and time, and interactions between these factors. Using the standard error of difference (SED) between the means for the treatment combinations on the residual degrees freedom (df) from ANOVA, least significant difference (LSD) at the 5% level was calculated for comparisons. For details, see 3.3.1 of Appendix I: Statistical analysis.

3.3.2. Experiment with water-hatched *G. pallida* J2s

In order to investigate whether spontaneously hatched (water-hatched, rather than PRD-hatched) *G. pallida* J2s would respond differently to the treatments, smaller numbers of the J2s that hatched in tap water were subjected to the same assays described in section 3.3.1 above. For this set of assays, tap water was used instead of high purity water for the treatment preparation. The statistical analysis was made as described in section 3.3.1 above. For details, see 3.3.2 of Appendix I: Statistical analysis.

3.3.3. Statistical analysis of combined data from 3.3.1 and 3.3.2

In order to test the effect of hatching preconditions (termed "origin"), *i.e.* whether *G. pallida* J2s were hatched in PRD or water had an effect, further analysis of combined data from the two experiments 3.3.1 and 3.3.2 was made. This involved a four-way ANOVA to test all combinations of "origin", treatment, area and time. Following ANOVA relevant means were compared using the least significant difference (LSD) value at the (P = 0.05) level of significance, calculated from the standard error of the difference (SED) on the residual degrees of freedom (df) from the ANOVA. For details, see 3.3.3 of Appendix I: Statistical analysis.

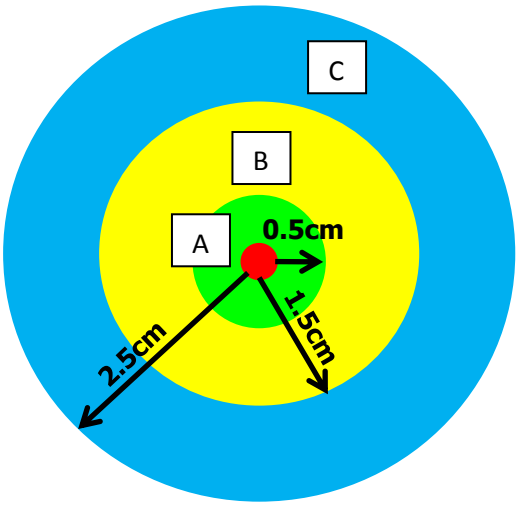


Figure 3.4. Motility assay. Number of *Globodera pallida* J2s in four different areas was counted at 30, 60 and 90 min: inner most circle in red, area A in light green, area B in yellow and area C in blue.

3.4. Invasion and development of *G. pallida* in *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

3.4.1. Invasion bioassays

3.4.2. Development bioassays

Plant materials

After emergence, plantlets of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium* (grown in the way described in section 2.2 of Chapter 2. General Materials and Methods) were planted individually in 6 × 6 cm pots with weed mix and grown at 20°C with 16:8 hours light:dark.

Invasion/development assays

Two to three-week-old plants were inoculated with > 200 *G. pallida* J2s (J2s suspended in 1 ml water were added to three holes made in the soil around the roots, and then these holes were covered with soil) and left at 19 °C (day) / 17 °C (night) with 16:8 hours light:dark, with three replicates for each of four time points for each plant species. The plants were washed free of soil at 1, 2, 3 and 4 days post inoculation (dpi) for the invasion assays, and at 5, 7, 10 and 14 dpi for the development assays. The pots of all the replicates of both plant species were positioned randomly. At each time point, the roots were weighed and stained with acid fuchsin according to the protocol described by Byrd *et al*. (1983), and nematode infection was examined, using a Wild M5 stereo-microscope. Further observations on development were made using one

S. sisymbriifolium plant at each of 3, 4, 5, 6, 7 and 10 weeks. From four weeks onwards, the water residue of the soil from a pot collected in a 250 µm and a 10 µm sieve was examined for the presence of adult females/cysts and adult males, respectively. For the extraction of adult males, the residue collected in a 10 µm sieve was placed on tissue paper (moist with water) on a plastic mesh, which was then immersed in water for 24 h to allow adult males, if any, to migrate to the water for examination under a microscope. The illustrations by Raski (1950) and Chitwood & Buhrer (1946) were used for identification of the developmental stages.

Statistical analysis

Count data (on the log scale) from the invasion assays were analysed by two-way ANOVA to assess the significance of plant species, time and the interaction of these two factors. Following ANOVA, relevant means were compared using the least significant difference (LSD) value at the (P = 0.05) level of significance, calculated from the standard error of the difference (SED) on the residual degrees of freedom (df) from the ANOVA. For details, see 3.4.1 of Appendix I: Statistical analysis.

Results

3.1. Movement of *G. pallida* towards the roots

3.1.1. Separate assays for individual plant species (arrestment/attraction assays)

3.1.1.1. Comparison between *S. tuberosum* L. cv. Desiree (potato, host) and *S. sisymbriifolium* (trap crop)

The results showed that within 30 min after the plantlets were placed in the Petri dishes, approximately 80% of the *G. pallida* J2s were already within the *a priori* "arrestment/attraction zone" of the roots of both *Solanum* species (Figures 3.5 and 3.6). As explained under Materials and methods, the zone was so named, because the J2s were randomly distributed to begin with and the number of J2s found in the zone was recorded, not their directions, and therefore, J2s might have arrived in the zone as a result of a modification to their speed or rate of non-directional kinesis* reactions to allelochemicals from the root (arrestment) or directed taxis* towards the root (attraction). For controls of both species only *ca*.10%, or less, of the J2s were in the zone at any of the time points.

Statistical analysis showed that there was no significant difference between the two *Solanum* species in arrestment/attraction of the J2s by/to their roots (P = 0.131, F-test). There was also no significant species-time interaction (P = 0.328, F-test). However, there was a significant effect of time (P < 0.001, F-test; means (natural logs) were: -0.142 (0 min), 2.178 (30 min), 2.325 (60

* See Glossary for definition.

min) and 2.386 (120 min), SED = 0.1442, 24 df, LSD (5%) = 0.2977 for comparison of these means on the log scale). For details, see 3.1.1.1 of Appendix I: Statistical analysis.

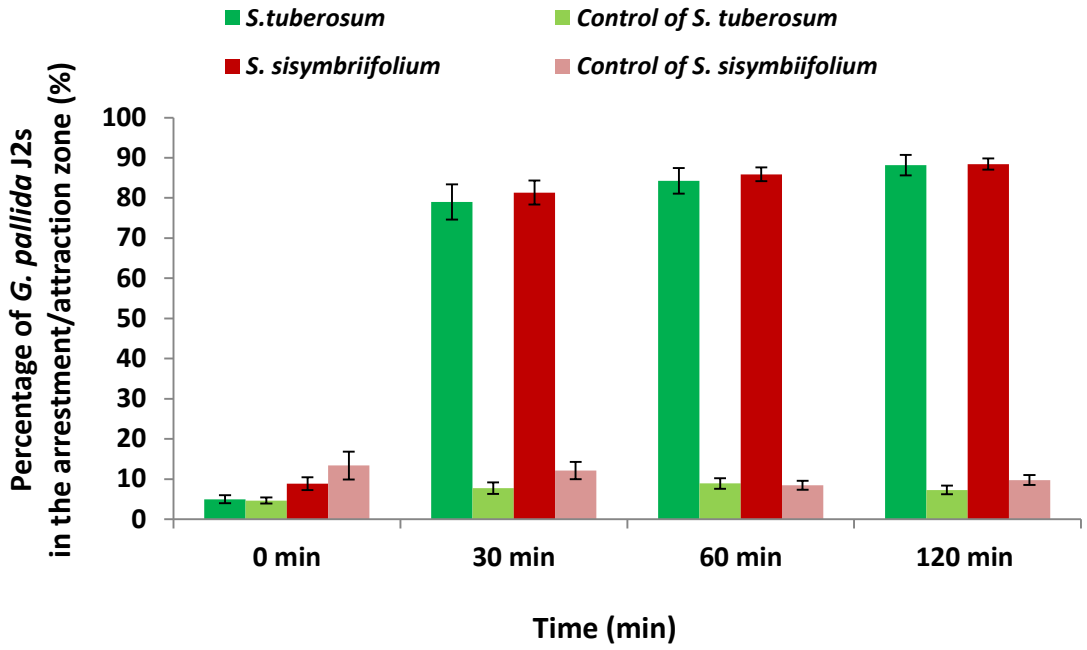


Figure 3.5. The mean (*n* = 5) and standard error of the percentage of *Globodera pallida* J2s that were in the arrestment/attraction zone at 0, 30, 60 and 120 min after the plantlets were placed in the Petri dishes. There was no significant difference (*P* = 0.131, F-test) between *Solanum tuberosum* Desiree and *S. sisymbriifolium* for arrestment/attraction of the J2s.

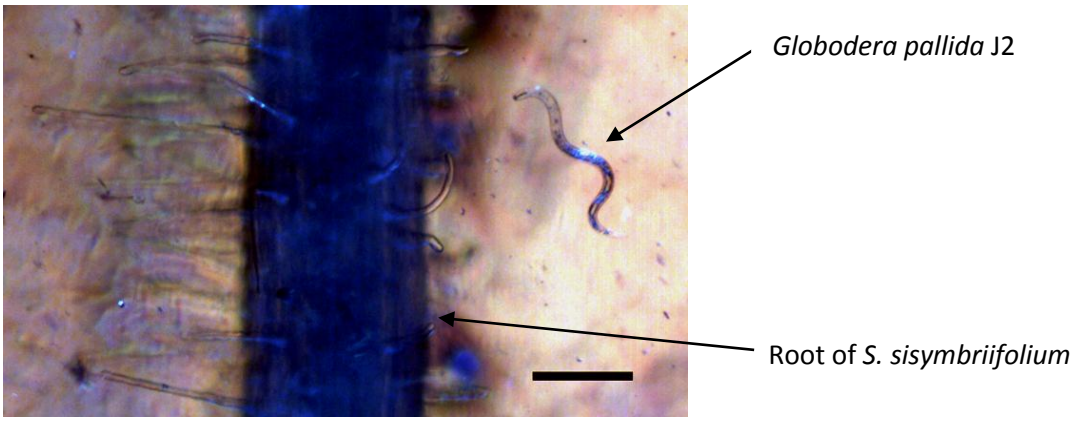


Figure 3.6. *Globodera pallida* J2 approaching the root of *Solanum sisymbriifolium.* Bar = 100 µm.

3.1.1.2. Comparison between *S. sisymbriifolium* (trap crop) and wheat (*Triticum aestivum*, non-host)

Globodera pallida J2s were arrested/attracted by/to the roots of wheat, a known non-host plant species, as much as to the trap crop, *S. sisymbriifolium,* with approximately 70% of the J2s already within the arrestment/attraction zone of the roots of both plant species within 30 min (Figure 3.7).

The roots of the seedlings used for the assays were stained at different time points after the assays: 2, 6, 8 and 10 days, with acid fuchsin to see if there was any invasion by the J2s. With wheat, only one J2 was found at 8 days out of four root samples tested, that is the mean number of J2s that invaded the root of wheat was 0.25, whereas with *S. sisymbriifolium* many J2s were found in every sample with a mean number of 21 (Figure 3.8).

Statistical analysis showed that there was no significant difference between the two plant species in arrestment/attraction of the J2s by/to their roots (P = 0.655, F-test). Also there was no significant species-time interaction (P = 0.614, F-test). However, there was an overall significant effect of time (P < 0.001, F-test; means (natural logs) were: -0.074 (0 min), 1.400 (30 min), 1.526 (60 min) and 1.555 (120 min), SED = 0.0992, 18 df, LSD (5%) = 0.2085 for comparison of these means on the log scale). For details, see 3.1.1.2 of Appendix I: Statistical analysis.

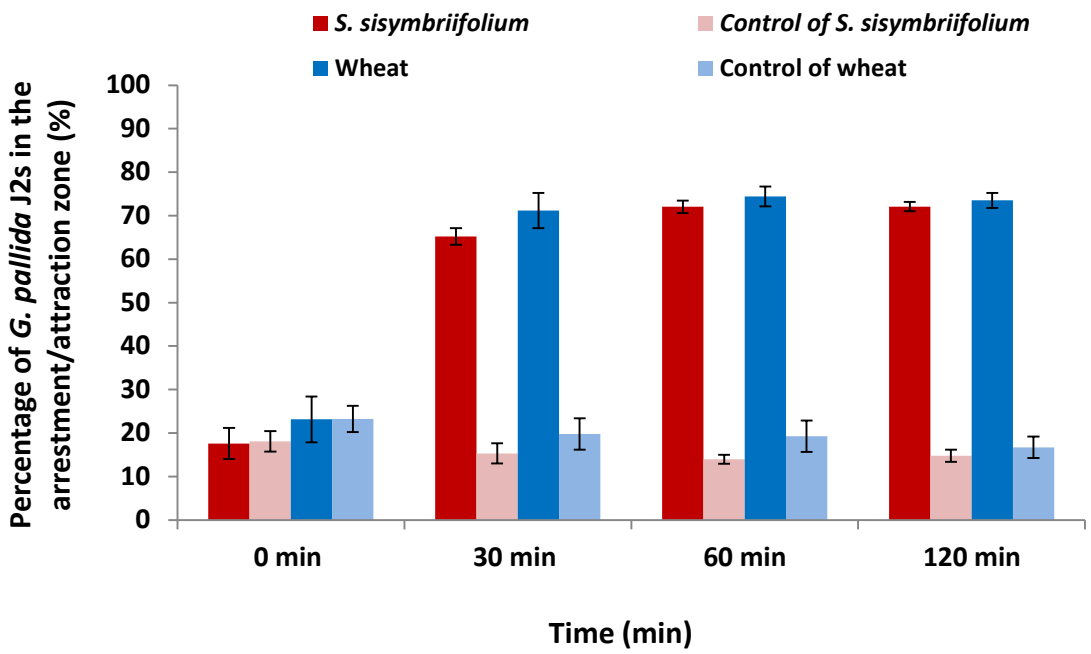
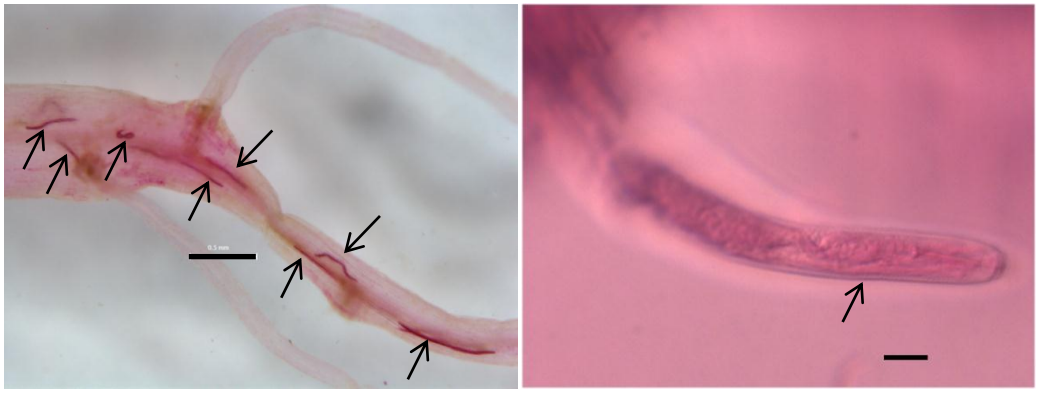


Figure 3.7. The mean (n = 4) and standard error of the percentage of *Globodera pallida* J2s that were in the arrestment/attraction zone at 0, 30, 60 and 120 min after the seedlings were placed in the Petri dishes. There was no significant difference (P = 0.655, F-test) between *Solanum sisymbriifolium* and wheat for arrestment/attraction of the J2s.



(a) (b)

Figure 3.8. Photographs of J2(s) (*arrow*) at 8 days after the arrestment/attraction assays in the roots of: (a) *Solanum sisymbriifolium* with many J2s (Bar = 0.5 mm), (b) wheat with the single J2 that was found out of four root samples tested. (Bar = 10 µm).

3.1.2. Choice assays between two plant species (attraction assays)

3.1.2.1. Between *S. tuberosum* L. cv. Desiree (host) and *S. sisymbriifolium* (trap crop)

Globodera pallida J2s were attracted (with directed orientation, taxis) in equal numbers to the roots of the two *Solanum* species with no overall main effect of plant species (P = 0.393, F-test) (Figure 3.9). However, there was a significant interaction between plant species, distance and time (P = 0.005, F-test). The δ-coefficients from the GLM indicated faster dispersion towards *S. tuberosum* Desiree for those J2s moving in that direction (δ-coefficients: 0.01454 (SE 0.00553) and 0.01204 (SE 0.00608) for *S. tuberosum* Desiree and *S. sisymbriifolium*, respectively). For statistical details, see 3.1.2.1 of Appendix I: Statistical analysis.

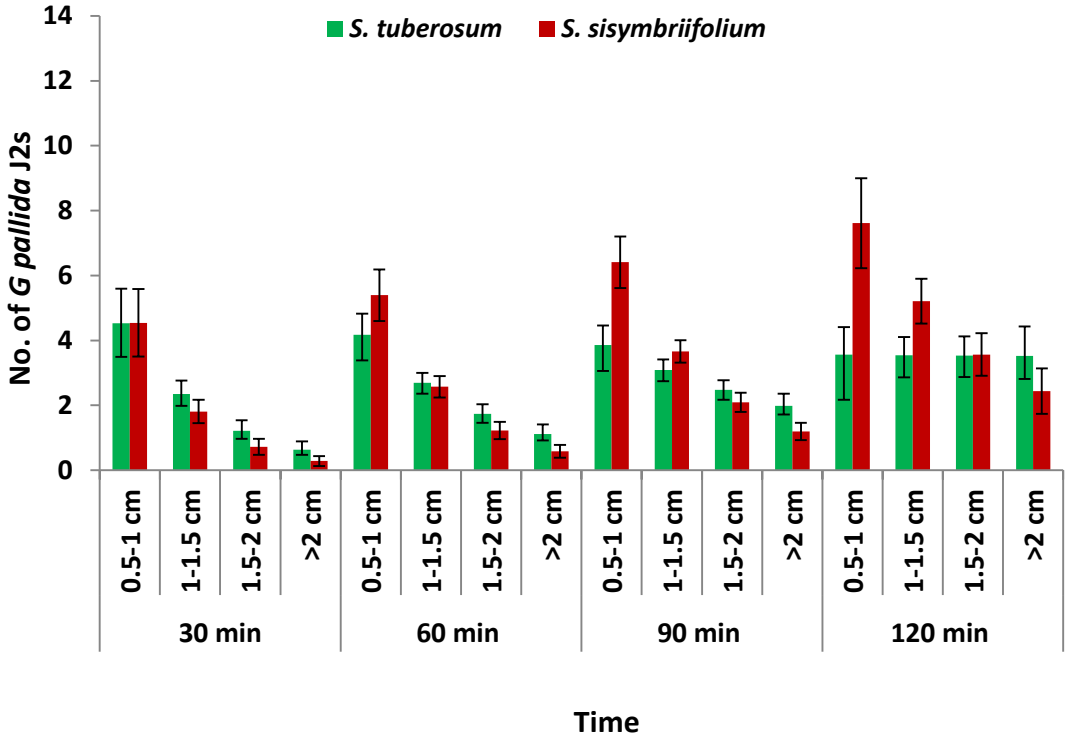


Figure 3.9. The predicted counts of *Globodera pallida* J2s in four zones measuring from the centre, 0.5-1 cm, 1-1.5 cm, 1.5-2 cm, and > 2 cm where a plant resides, at each time point with standard error of prediction (n = 3) for the choice between *Solanum tuberosum* Desiree and *S. sisymbriifolium*. There was no overall main effect of plant species (P = 0.393, F-test) but a significant interaction between plant species, distance and time (P = 0.005, F-test): the δ-coefficients from the GLM indicated that the juveniles moved faster towards *S. tuberosum* Desiree. See Appendix II for raw data means and standard errors.

3.1.2.2. Between *S. tuberosum* L. cv. Desiree (host) and wheat (non-host)

Significantly more nematodes went towards wheat with an overall main effect of plant species (P = 0.010, F-test) (Figure 3.10). However, there was also a significant interaction between plant species, distance and time (P < 0.001, F-test), and those nematodes that moved in the *S. tuberosum* Desiree direction appeared to be moving slightly faster (δ-coefficient: 0.01123 (SE 0.00350)) than those that moved to wheat (δ-coefficient: 0.01089 (SE 0.00308)). For statistical details, see 3.1.2.2 of Appendix I: Statistical analysis.

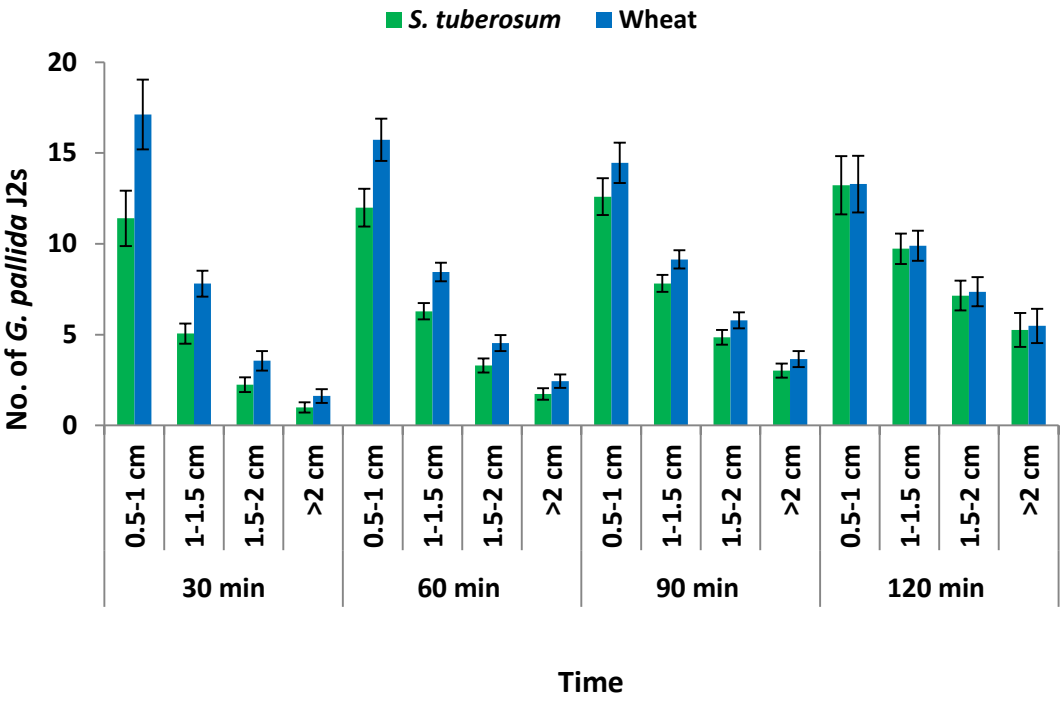


Figure 3.10. The predicted counts of *Globodera pallida* J2s in four zones measuring from the centre, 0.5-1 cm, 1-1.5 cm, 1.5-2 cm, and > 2 cm where a plant resides, at each time point with standard error of prediction (n = 3) for the choice between *Solanum tuberosum* Desiree and wheat. Significantly more nematodes went towards wheat with an overall main effect of plant species (P = 0.010, F-test). However, there was also a significant interaction between plant species, distance and time (P < 0.001, F-test): the δ-coefficients from the GLM indicated that juveniles moved slightly faster towards *S. tuberosum* Desiree. See Appendix II for raw data means and standard errors.

3.1.2.3. Between *S. sisymbriifolium* (trap crop) and wheat (non-host)

In the choice between *S. sisymbriifolium* and wheat, the J2s were attracted to both plant species equally with no overall main effect of plant species (P = 0.970, F-test). There was a significant interaction between plant species, distance and time (P = 0.041, F-test) (Figure 3.11). The δ-coefficients from the GLM indicated faster movement towards *S. sisymbriifolium* for those nematodes moving in that direction (δ-coefficients: 0.01306 (SE 0.00693) and

0.01062 (SE 0.00635) for *S. sisymbriifolium* and wheat, respectively). For statistical details, see 3.1.2.3 of Appendix I: Statistical analysis.

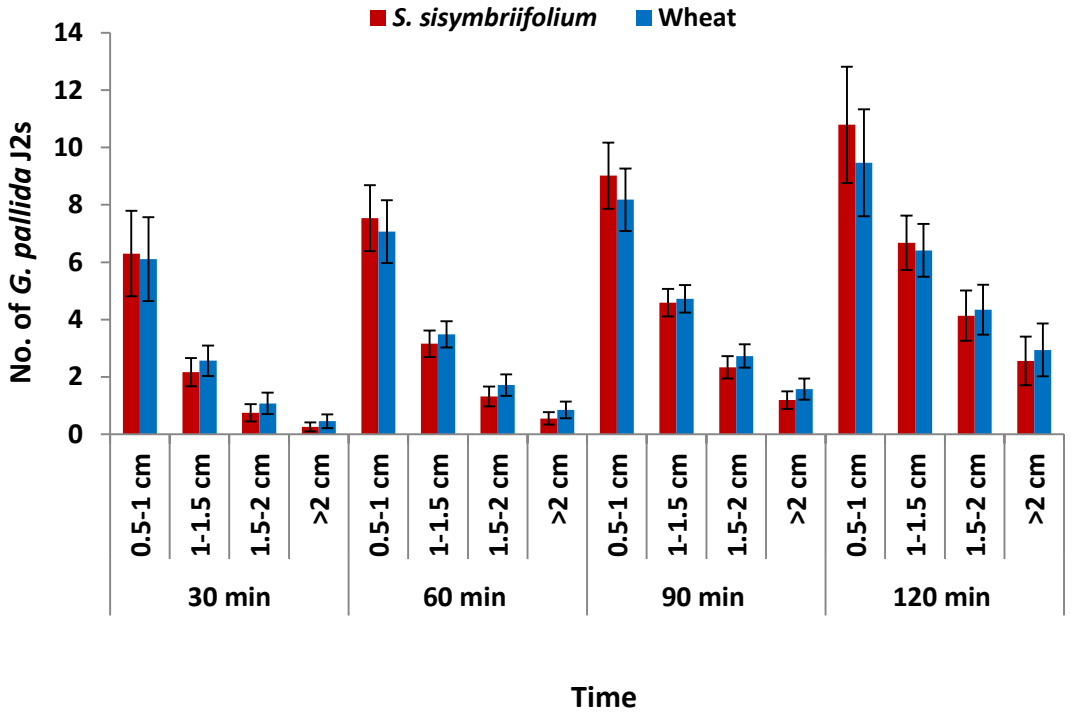


Figure 3.11. The predicted counts of *Globodera pallida* J2s in four zones measuring from the centre, 0.5-1 cm, 1-1.5 cm, 1.5-2 cm, and > 2 cm where a plant resides, at each time point with standard error of prediction (n = 3) for the choice between *Solanum sisymbriifolium* and wheat. There was no overall main effect of plant species (P = 0.970, F-test) but there was a significant interaction between plant species, distance and time (P = 0.041, F-test): the δ-coefficients from the GLM indicated that the juveniles moved faster towards *S. sisymbriifolium*. See Appendix II for raw data means and standard errors.

3.1.2.4. Between *S. tuberosum* L. cv. Desiree (host) and "no plant"

When the J2s were given a choice between *S. tuberosum* Desiree and no plant, significantly more J2s moved towards *S. tuberosum* Desiree, with an overall main effect of plant species (P < 0.001, F-test) (Figure 3.12). There was also a significant interaction between plant species, distance and time (P <

0.001, F-test). Those J2s that moved in the no plant direction, however, appeared to be moving faster (δ-coefficient: 0.02606 (SE 0.00919)) than those that moved to *S. tuberosum* Desiree (δ-coefficient: 0.02095 (SE 0.00598)). For statistical details, see 3.1.2.4 of Appendix I: Statistical analysis.

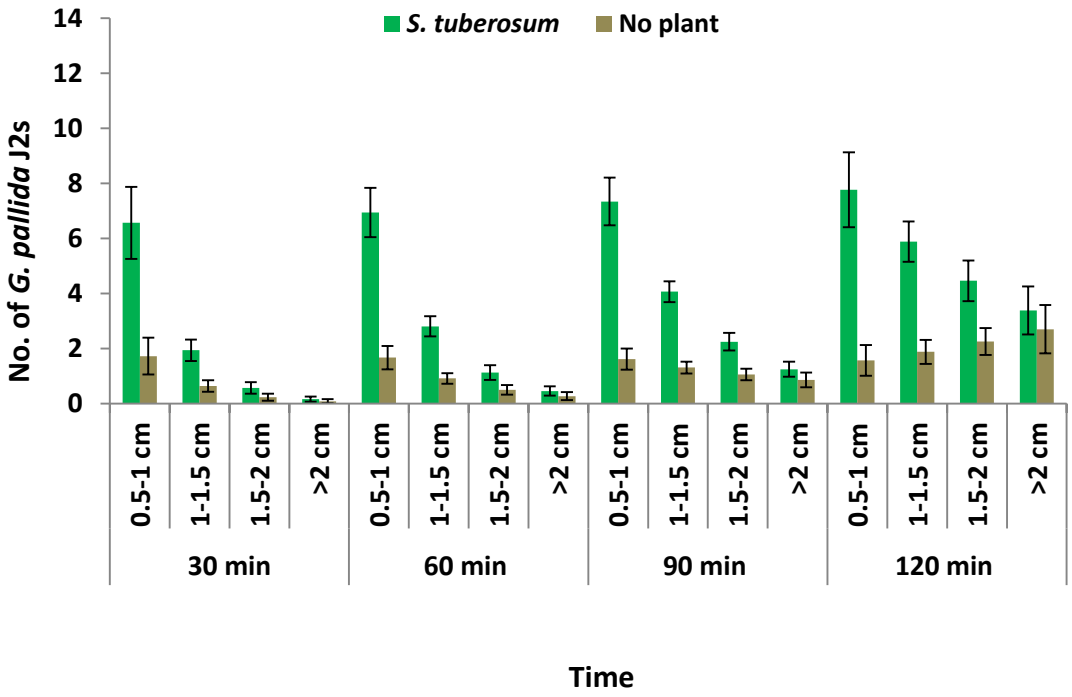
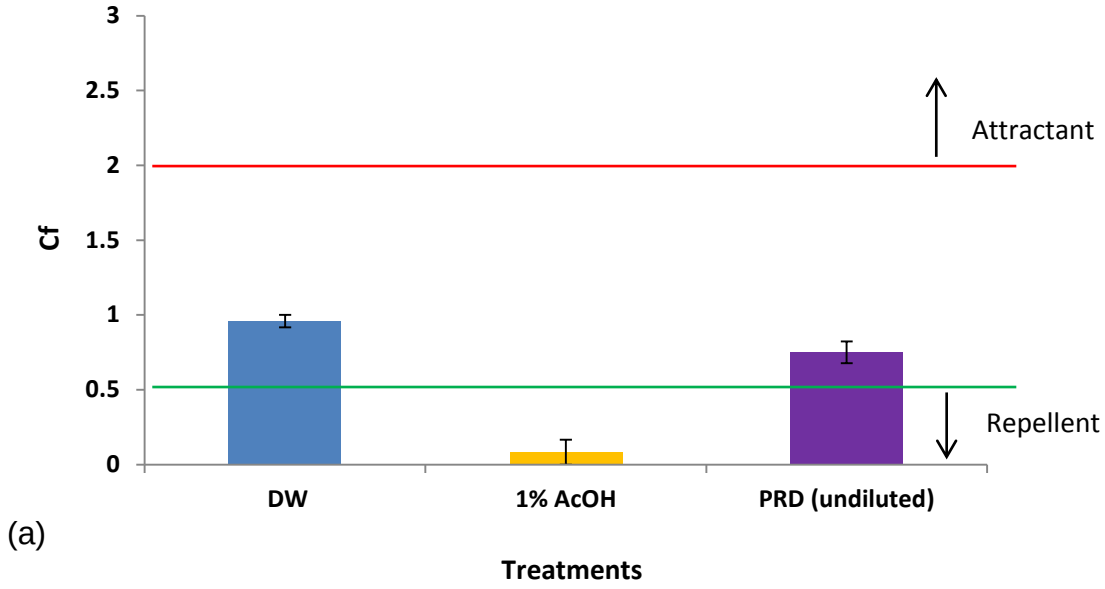


Figure 3.12. The predicted counts of *Globodera pallida* J2s in four zones measuring from the centre, 0.5-1 cm, 1-1.5 cm, 1.5-2 cm, and > 2 cm where a plant resides, at each time point with standard error of prediction (n = 3) for the choice between *Solanum tuberosum* Desiree and no plant. There was an overall main effect of plant species (P < 0.001, F-test). There was also a significant interaction between plant species, distance and time (P < 0.001, F-test): the δ-coefficients from the GLM indicated that the juveniles moved faster in the no plant direction. See Appendix II for raw data means and standard errors.

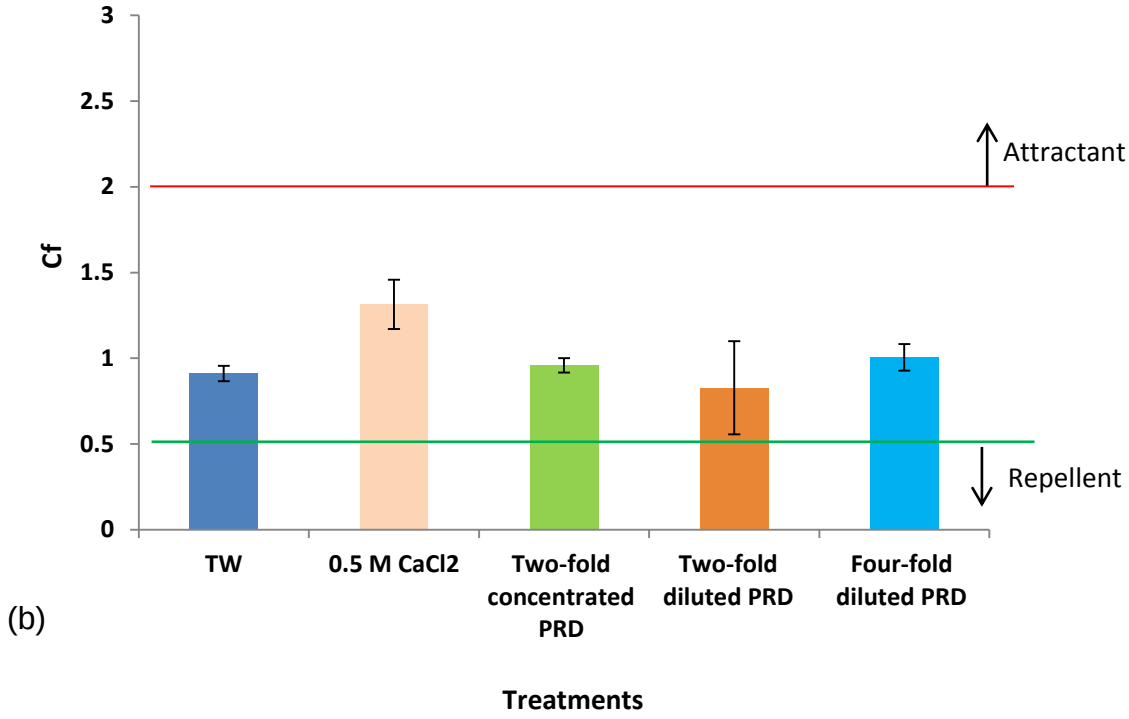
3.2. Chemotaxis of *G. pallida* towards root exudates of *S. tuberosum* L. cv. Desiree

The results showed that undiluted PRD was neutral (0.5 ≤ Cf ≤2.0), which means that undiluted PRD itself would not attract *G. pallida* J2s. However, 1% acetic acid (AcOH) was repellent (Cf < 0.5), and there was no effect with distilled water (DW) (0.5 ≤ Cf ≤ 2.0) (Figure 3.13a). Statistical analysis showed that there was a significant difference between the treatments (P < 0.001, F-test) with both DW and undiluted PRD being significantly different from 1% AcOH (P < 0.05, LSD).

0.5 M $CaCl_2$ was tested as a positive control (an attractant) following the study by Wuyts *et al*. (2006), against tap water (TW), two-fold concentrated PRD, two-fold TW-diluted PRD and four-fold TW-diluted PRD. All five treatments were neutral (0.5 ≤ Cf ≤ 2.0) (Figure 3.13b). Statistical analysis showed that there was no significant difference between treatments (P = 0.237, F-test). Wuyts *et al.* (2006) reported that 0.5 M $CaCl_2$ worked as an attractant (Cf > 2) in 50 to 70 % of the experiments with burrowing nematode (*Radopholus similis* Cobb), root-lesion nematode (*Pratylenchus penetrans* (Cobb) Filipjev & Schuurmans Stekhoven) and *Meloidogyne incognita.* In the current study, although 0.5 M $CaCl_2$ was neutral, there was marginally significant difference between 0.5 M $CaCl_2$ (mean 1.314) and two-fold diluted PRD (mean 0.828) with SED = 0.2045 on 10 df, LSD (5%) = 0.4556. For statistical details see 3.2 of Appendix I: Statistical analysis.



(a)



(b)

Figure 3.13. The mean (*n* = 3) and standard error of chemotaxis factors (Cf) for each of the treatments tested. (a) For distilled water (DW), 1% acetic acid (AcOH) and undiluted PRD. DW and undiluted PRD were found to be neutral, with 1% AcOH being a repellent. SED = 0.0962, 6 df, LSD (5%) = 0.2355 for comparison of means, (b) for tap water (TW), 0.5 M $CaCl_2$, two-fold concentrated PRD, two-fold diluted PRD and four-fold diluted PRD. All treatments were neutral. SED = 0.2045, 10 df, LSD (5%) = 0.45560 for comparison of means.

3.3. Motility assays with root exudates of *S. tuberosum* L. cv. Desiree

3.3.1. Experiment with PRD-hatched *G. pallida* J2s

Globodera pallida J2s that hatched in PRD responded to water and PRD treatments in a similar way, gradually moving further from the centre with time. The means (on the raw scale) of the percentage of J2s in each area are given in Figure 3.14a. Analysing the data on the logit scale, there was no overall effect of treatments (P = 0.803, F-test). There was no interaction between treatment and area (P = 0.824, F-test), treatment and time (P = 0.280, F-test), or treatment, area and time (P = 0.073, F-test). However, there was a significant interaction between area and time (P < 0.001, F-test; See Table 3.1), which indicates that, regardless of treatments, the J2s were found further away from the application site with time. For statistical details, see 3.3.1 of Appendix I: Statistical analysis.

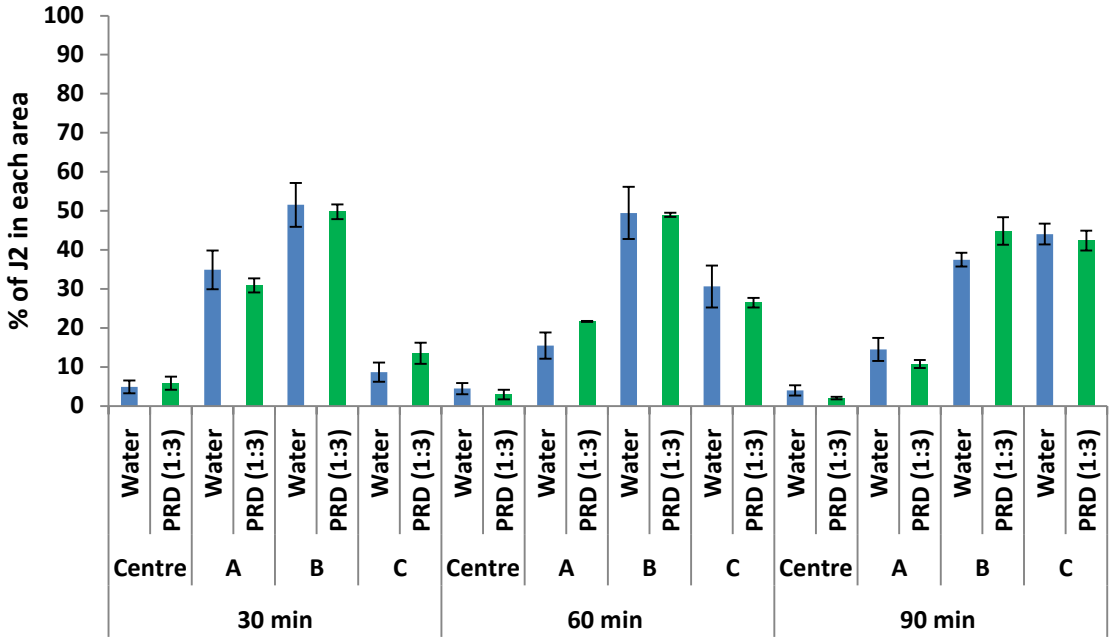
3.3.2. Experiment with water-hatched *G. pallida* J2s

As shown in Figure 3.14b, *Globodera pallida* J2s that hatched in water also did not respond differently to treatments of PRD or water with no effect of treatments (P = 0.934, for data on the logit scale, F-test). Similarly to results in section 3.3.1, there was no interaction between treatment and area (P = 0.765, F-test), treatment and time (P = 0.765, F-test) or treatment, area and time (P = 0.278, F-test). However, there was a significant interaction between area and time (P < 0.001, F-test; See Table 3.2), which indicates that the J2s were found

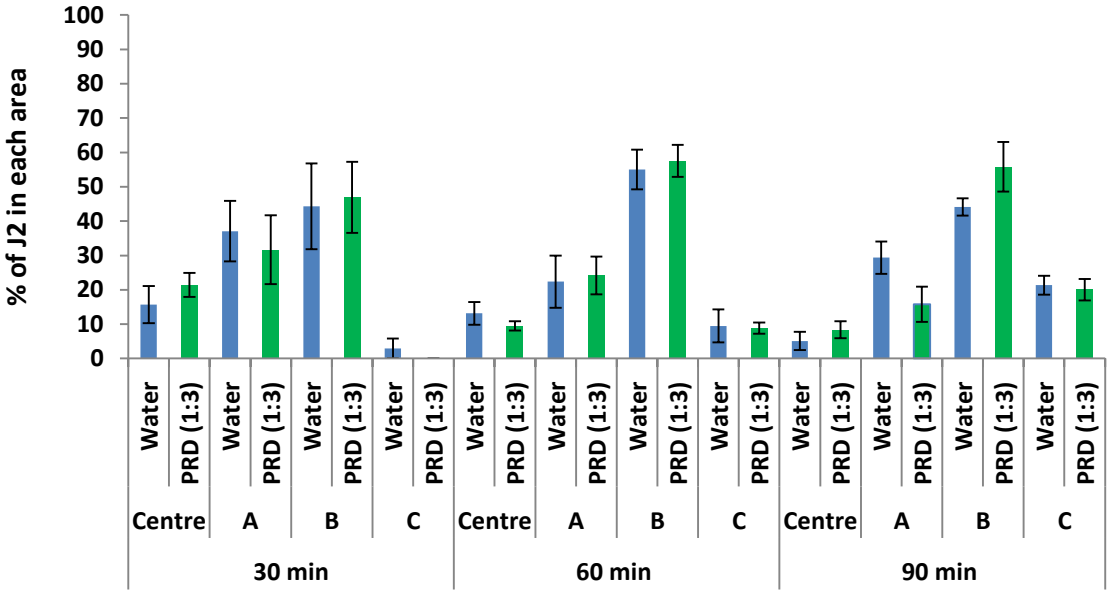
further away from the application site at the centre with time, without being affected by the treatments. For statistical details, see 3.3.2 of Appendix I: Statistical analysis.

3.3.3. Statistical analysis of combined data from 3.3.1 and 3.3.2

In order to examine if hatching in PRD or water (termed “origin”) made any difference in movements, the data from both experiments (3.3.1 and 3.3.2) were analysed together on the logit scale. Although there was no overall effect of origin (P = 0.583, F-test) or treatments (P =0.993, F-test), there were significant interactions between area and “origin” (P < 0.001, F-test) (Figure 3.15) as well as between area and time (P < 0.001, F-test) (Figure 3.16), which indicates that PRD-hatched J2s moved out more from the centre and also moved further compared with water-hatched J2s. For statistical details, see 3.3.3 of Appendix I: Statistical analysis.



(a)



(b)

Figure 3.14. The mean (n = 3) and standard error of the percentage of *Globodera pallida* J2s in each area of a dish at three time points (30, 60 and 90 min). (a) 3.3.1 (PRD-hatched J2s), no overall effect of treatments (P = 0.803, for data on logit scale, F-test), (b) 3.3.2 (water-hatched J2s), no overall effect of treatments (P = 0.934, for data on logit scale, F-test).

Area	Time (min)		
	30	60	90
0 (Centre)	-3.005[a]	-3.444[b]	-3.615[b]
0.5 (A)	-0.723[a]	-1.517[b]	-1.973[c]
1.5 (B)	0.026[a]	-0.034[a]	-0.361[b]
2.5 (C)	-2.165[a]	-0.932[b]	-0.274[c]

Table 3.1. Area by time interaction means table on the logit scale for 3.3.1 (PRD-hatched J2s). Within areas SED = 0.1917, 32 df, LSD (5 %) = 0.3905; all other comparisons SED = 0.2634, 25 df, LSD (5 %) = 0.5415. Means across time within an area that are significantly different ($P < 0.05$, LSD) are indicated with different letters.

Area	Time (min)		
	30	60	90
0 (Centre)	-1.512[a]	-2.037[a]	-2.755[b]
0.5 (A)	-0.683[a]	-1.257[a]	-1.32[a]
1.5 (B)	-0.185[a]	0.251[a]	0.002[a]
2.5 (C)	-4.222[a]	-2.487[b]	-1.324[c]

Table 3.2. Area by time interaction means table on the logit scale for 3.3.2 (water-hatched J2s). Within areas SED = 0.2423, 32 df, LSD (5 %) = 0.6979; all other comparisons SED = 0.3074, 29 df, LSD (5 %) = 0.8886. Means across time within an area that are significantly different ($P < 0.05$, LSD) are indicated with different letters.

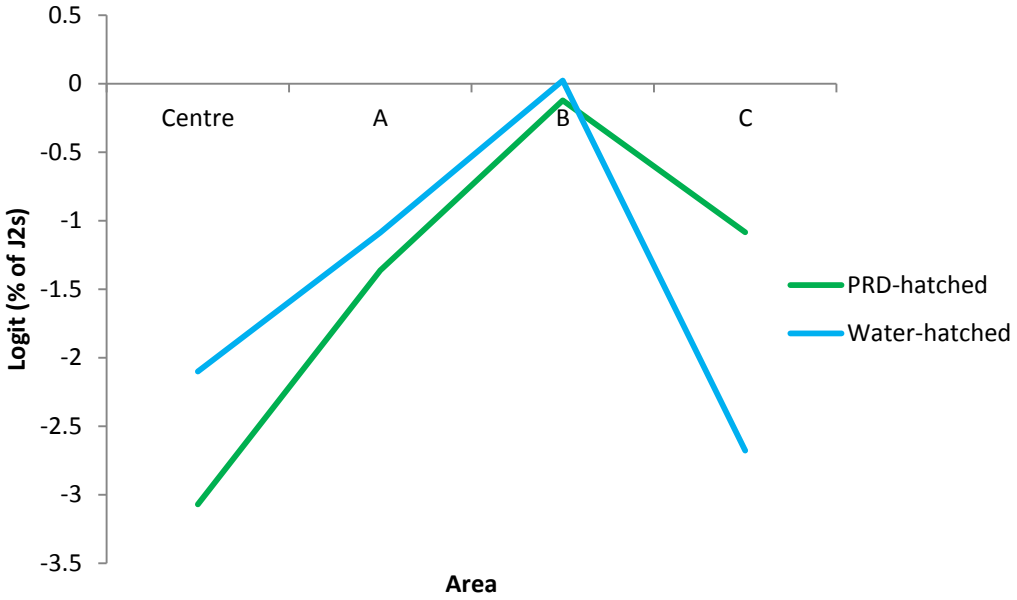


Figure 3.15. Interaction between "origin" and area, *i.e.* averaging over time. There were more J2s in C when using PRD-hatched J2s than using water-hatched J2s. For comparisons: within an "origin" SED = 0.2664, 24 df, LSD (5 %) = 0.5457; all other comparisons SED = 0.2462, 29 df, LSD (5 %) = 0.5029.

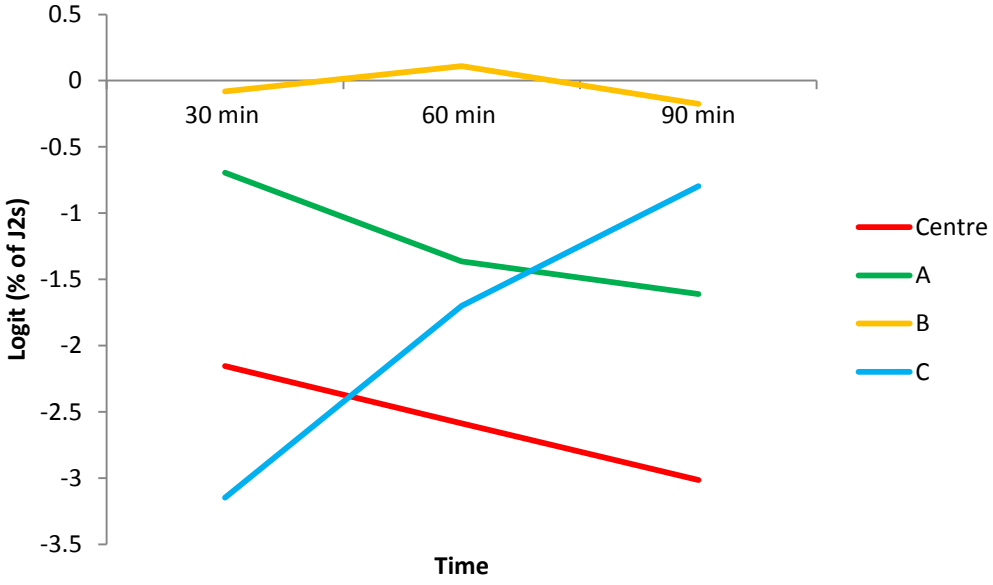


Figure 3.16. Interaction between area and time, *i.e.* averaging over "origin". With time, large increase of J2s in C, gradual decrease in Centre and A, hardly any change in B. For comparisons: within an area SED = 0.1927, 64 df, LSD (5%) = 0.3850; all other comparisons SED = 0.2443, 58 df, LSD (5%) = 0.4889.

3.4. Invasion and development of *G. pallida* in *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

3.4.1. Invasion bioassays

Globodera pallida J2s invaded the roots of *S. sisymbriifolium* in large numbers from 1 day post inoculation (dpi). The actual count of the J2s in the roots was much larger in *S. tuberosum* Desiree than in *S. sisymbriifolium* at 1 and 4 dpi (Figure 3.17), but when the weight of roots (Figure 3.18) was taken into account, the number of the J2s per gram root of *S. sisymbriifolium* exceeded that of *S. tuberosum* at every time point: 1, 2, 3 and 4 dpi (Figure 3.19 on the natural log scale). Analysing these latter data (on the natural log scale), the overall difference between plant species was significant ($P < 0.001$, F-test), along with the main effect of time ($P = 0.001$, F-test). The interaction was also marginally significant ($P = 0.047$, F-test), indicating that the trend of infection over time was slightly different for the two plant species; infection in *S. sisymbriifolium* increased steadily to 3 dpi, whereas it fluctuated in *S. tuberosum* Desiree. For statistical details, see 3.4.1 of Appendix I: Statistical analysis.

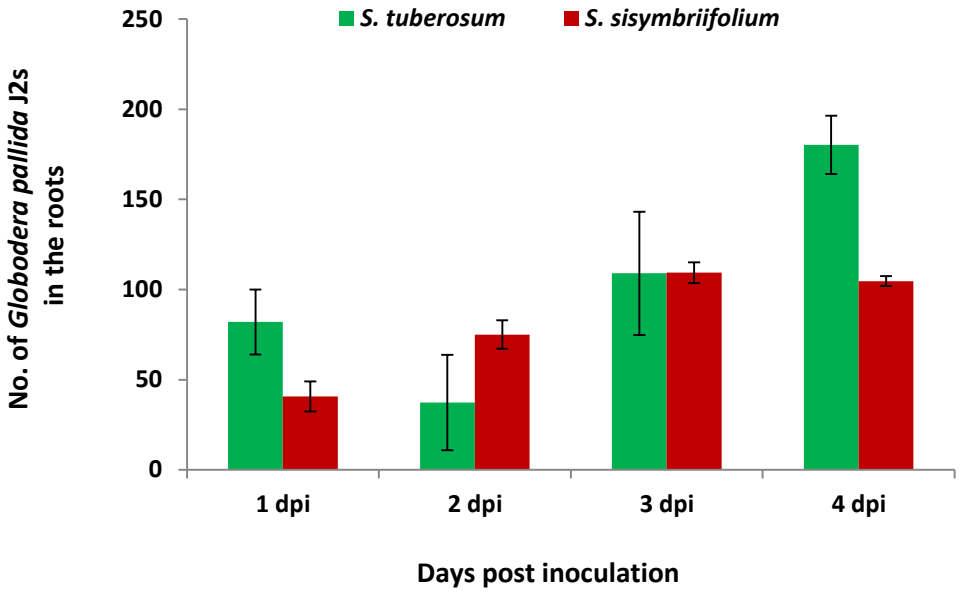


Figure 3.17. The mean (*n* = 3) and standard error of the number of *Globodera pallida* J2s inside the roots of *Solanum tuberosum* Desiree and *S. sisymbriifolium* at 1, 2, 3 and 4 dpi.

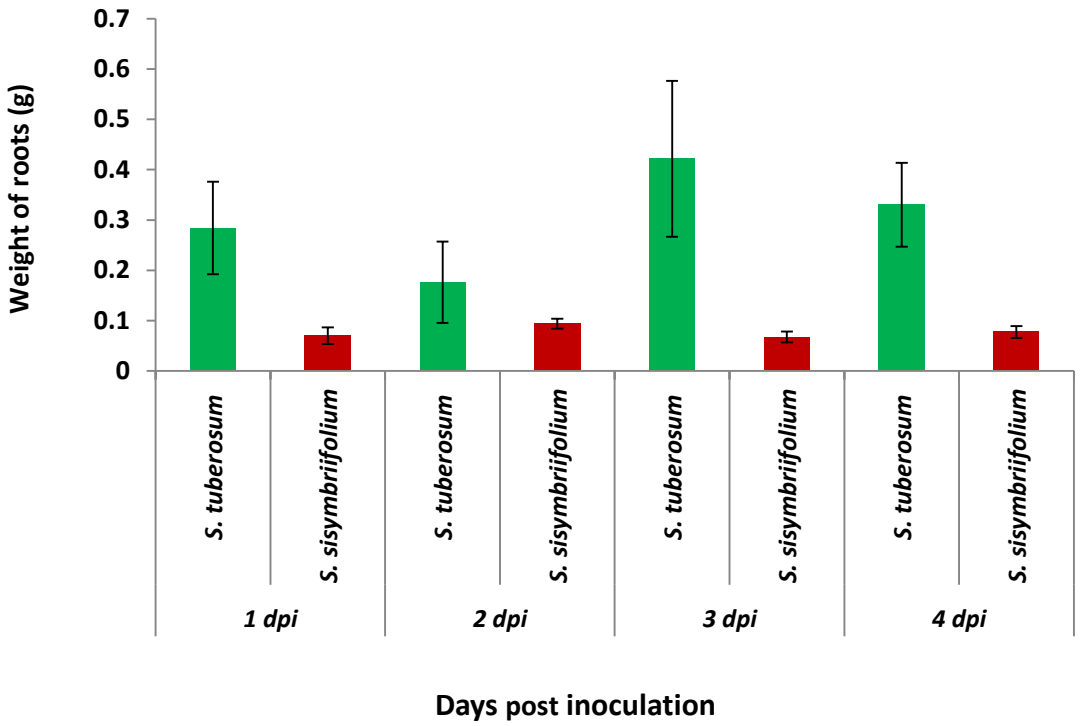


Figure 3.18. The mean (*n* = 3) and standard error of the weight (g) of the roots of *Solanum tuberosum* Desiree and *S. sisymbriifolium* at 1, 2, 3 and 4 dpi.

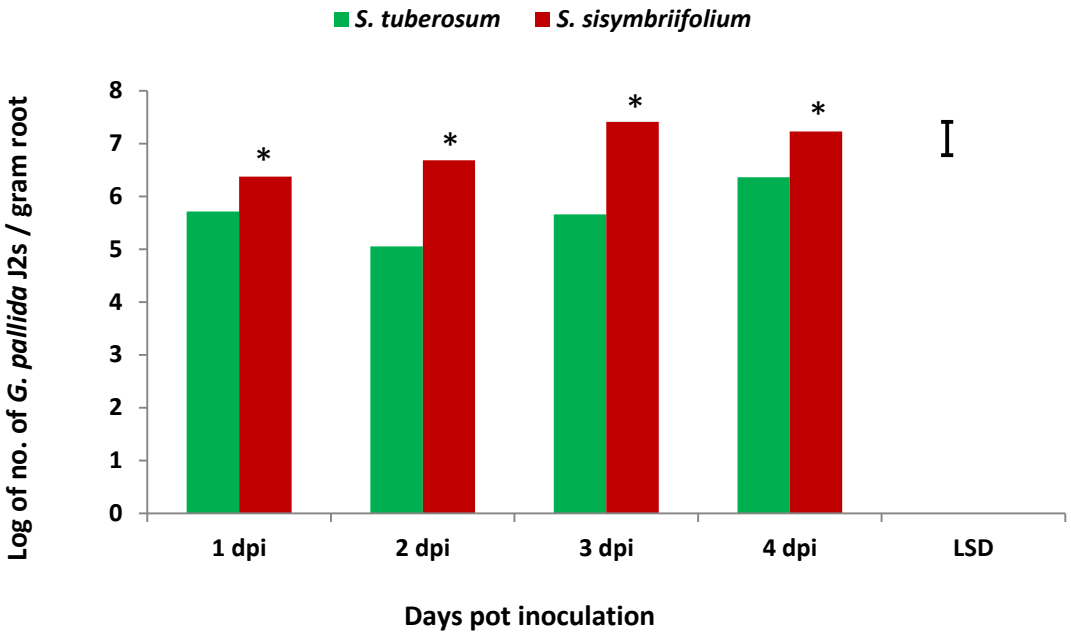


Figure 3.19. The mean natural log of number of *Globodera pallida* J2s per gram root of *Solanum tuberosum* Desiree and *S. sisymbriifolium* at 1, 2, 3 and 4 dpi (n = 3). The bar represents the LSD (5 %). There was a significant interaction between plant species and days post inoculation (P = 0.047, F-test), SED = 0.2983, 16 df, LSD (5 %) = 0.6323. The number of J2s/g root of *S. sisymbriifolium* (on the natural log scale) was significantly more than that of *S. tuberosum* Desiree (P < 0.05, LSD) at every time point, which is indicated with an asterisk. See Appendix II for raw data means and standard errors.

3.4.2. Development bioassays

Globodera pallida J2s inside the roots of *S. tuberosum* Desiree started to develop at 10 dpi and many third-stage juveniles (J3), fourth-stage juveniles (J4) and young females were observed by 14 dpi. However, no development further than J2 was found inside the roots of *S. sisymbriifolium* at 14 dpi (Figure 3.20), except for one J3 and one slightly more developed J2, in all of the plants examined. In order to see if further development would occur at a later stage, observations were made at 3, 4, 5, 6, 7 and 10 weeks post inoculation (wpi) in

one *S. sisymbriifolium* plant at each of these time points. Only J2s were found at 3 and 4 wpi, and at 5 wpi just one moulting J3 was observed (Figure 3.21a) with very few other J3. At 5 wpi, a few J2s were still observed, but at 6 wpi no nematodes were found, with only one nematode (J3, Figure 3.21b) in the roots at 7 wpi. At 10 wpi, no nematodes were seen in the roots. No adult females/cysts or adult males were found in the soil.

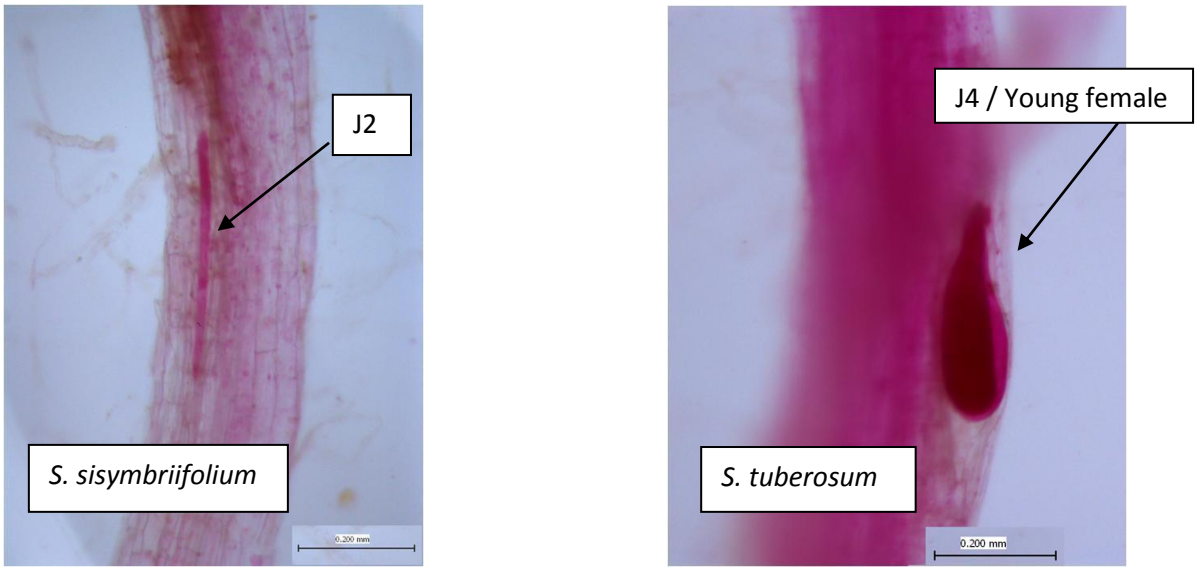
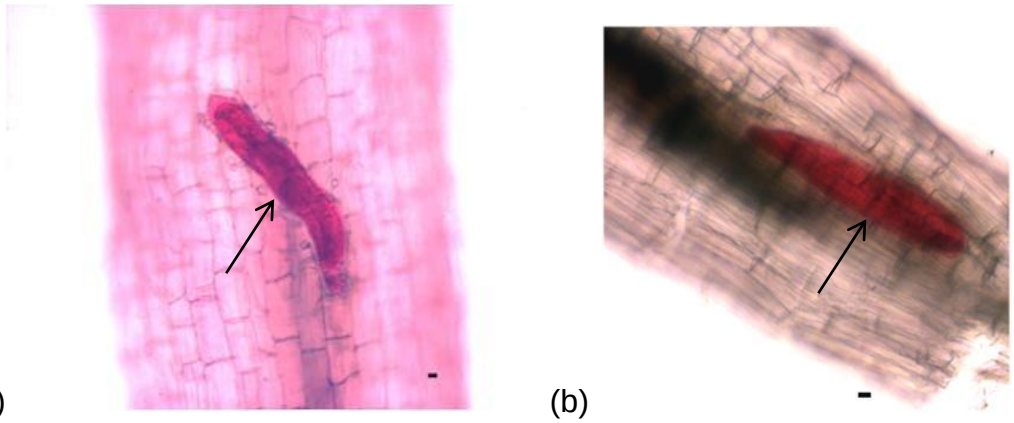


Figure 3.20. Photographs of infected roots stained with acid fuchsin. Roots were infected with *Globodera pallida* J2s and observed at 14 dpi. The J2s developed to J4s/young females in roots of *Solanum tuberosum* Desiree, whereas in roots of S*. sisymbriifolium*, they did not develop further. (Bar = 0.2 mm).



(a) (b)

Figure 3.21. Developed nematodes (*arrow)* in the root of *S. sisymbriifolium*. (a) Moulting J3 at 5 wpi, (b) J3 at 7 wpi. (Bar = 10 µm).

Discussion

3.1. Movement of *G. pallida* towards the roots

The results of arrestment/attraction assays in section 3.1.1 revealed that *G. pallida* J2s were arrested/attracted by/to the trap crop, *S. sisymbriifolium* (Figure 3.5) and even by/to a known non-host, wheat (Figure 3.6), with no significant difference in the percentage of the J2s in the arrestment/attraction zone between the host (*S. tuberosum* Desiree) and the trap crop, nor between the trap crop and the non-host. Due to the particular set-up of these experiments, where the J2s were randomly distributed in a dish to begin with and directed movement of the J2s was not recorded, the results would not have necessarily constituted attraction, which demands participation of directed orientation reactions (taxis) to a source of stimulation. It is possible that the J2s arrived in the zone as a result of a modification to their speed or rate of non-directional reactions (kinesis) to allelochemicals from the root. Therefore, choice

assays (section 3.1.2) were conducted to clarify if the reactions of the J2s observed in arrestment/attraction assays in section 3.1.1 were truly attraction to the roots.

The results of choice assays revealed that *G. pallida* J2s that were placed between two plant species were attracted (with directed orientated taxis reactions) in equal numbers to the host (*S. tuberosum* Desiree) and the trap crop (*S. sisymbriifolium*) (Figure 3.9), and also to the trap crop (*S. sisymbriifolium*) and the non-host plant (wheat) (Figure 3.11). However, interestingly, they moved slightly faster towards *S. tuberosum* Desiree (host) than to *S. sisymbriifolium* (trap crop) (Figure 3.9), and also faster to *S. sisymbriifolium* (trap crop) than to wheat (non-host) (Figure 3.11). However, significantly more *G. pallida* J2s moved towards wheat than to *S. tuberosum* Desiree*,* but the speed of movement towards *S. tuberosum* Desiree was slightly faster (Figure 3.10). It is interesting that a larger number of J2s were attracted to their non-host plant, wheat, even though the speed was somewhat slower than to the host, *S. tuberosum* Desiree. When the J2s were given a choice between *S. tuberosum* Desiree and no plant, significantly more J2s moved towards *S. tuberosum* Desiree*,* but the small number of nematodes that moved in the no plant direction moved faster (Figure 3.12). It is possible that not all nematodes respond to a set of stimuli in the same way, with some more sensitive to repellent components of root allelochemicals (Prot, 1980). Or it can be speculated that when a large number of nematodes are placed together and there is only one host plant nearby, some nematodes may move away from the majority as a survival instinct and they have to move faster to be able to reach

another host which may be some distance away before their lipid reserves run out. In summary, these results of choice (attraction) assays in section 3.1.2 can be described in the following way, i) for overall number of the J2s that moved to a plantlet, Host = Trap crop, Host < Non-host, Trap crop = Non-host, Host > No plant, ii) for the speed of movement of the J2s towards a plantlet, Host > Trap crop > Non-host, Host < No plant.

The signals involved in attracting nematodes can be divided into long-distance* (to the root area – a scale of several cm), short-distance* (to individual host roots) and local attractants* (to preferred invasion sites) (Perry, 2005; Spence *et al.*, 2008). So far, the only long-distance attractant described for plant parasitic nematodes is carbon dioxide (CO_2) (reviewed in Curtis *et al*. (2009)), which is generic. However, Wang *et al*. (2009a) reported that attraction to CO_2 might be due to acidification of solutions by dissolved CO_2 rather than to CO_2 itself in the experiment with *Meloidogyne hapla*. Therefore, the involvement of CO_2 as a long-distance attractant for nematodes is currently inconclusive. Given the distance used for choice (attraction) assays in section 3.1.2, which was 2 cm from the nematode application site to the plantlet, it can be suggested that the assays tested short-distance attractants to *G. pallida*, rather than long-distance or local attractants. If so, it is possible that short-distance attractants are also something generic, rather than host-specific, as *G. pallida* J2s were attracted even to their non-host plant.

* See Glossary for definition.

There are reports that root-knot nematodes *Meloidogyne incognita* and *M. graminicola* Golden & Birchfield took the most direct route to their preferred hosts but the longest route towards the poor hosts (Reynolds *et al.*, 2011). Griffin (1969) also reported that significantly more *Meloidogyne hapla* J2s moved towards the susceptible cultivar in a choice between a resistant and a susceptible cultivar of alfalfa. Dalzell *et al*. (2011) also showed both *M. incognita* and *G. pallida* were strongly attracted to their respective host plants. Dalzell *et al*. (2011), however, tested the choice between the host and no plant, and between two different host plant species, but not the choice between the host and non-host. This current study is the first to include a non-host plant for attraction bioassays for PCN. This study, for the first time, demonstrated that *G. pallida* J2s do not necessarily choose their host plant strongly over the trap crop or a non-host plant, which seems to differ from *Meloidogyne* spp. Unlike *Meloidogyne* spp. and other sedentary endoparasitic nematodes, PCN needs a chemical cue (hatching factor, HF) exuded from its host roots for the majority to hatch, (Trudgill *et al.*, 1996; Jones *et al.*, 1998; Devine *et al.*, 1999), and therefore, after hatching occurs a plant nearby is likely to be their host. Armed with a sophisticated mechanism for hatching as a survival strategy, it is likely that PCN is not equipped with another sophisticated mechanism to distinguish between plant species, and therefore attraction might rely on general plant signals. So far, it has been demonstrated that PCN has a very narrow host range and relies on HF of solanaceous plants for hatching, but specific compounds involved in attraction have not been identified.

Of course, being attracted does not automatically mean that PCN invades the roots. While the long- and short-distance attractants for PCN could be generic, when it comes to local attractants, there must be host specificity. This was confirmed by staining the roots of the plantlets used for arrestment/attraction assays for the comparison between *S. sisymbriifolium* (trap crop) and wheat (non-host) in section 3.1.1.2. While wheat (non-host) had hardly any nematode in the roots (Figure 3.8b), many J2s had successfully penetrated the roots of the trap crop, *S. sisymbriifolium* (Figure 3.8a).

Even though the short-distance attractants for PCN may be generic, the difference in speed of *G. pallida* J2s towards plant species in choice (attraction) assays in section 3.1.2, *i.e.* Host > Trap crop > Non-host, may indicate that allelochemicals from the host *S. tuberosum* Desiree induce more positive orthokinesis* (relating to speed of non-directional movement) as well as taxis to the J2s than *S. sisymbriifolium* (trap crop) or wheat (non-host).

3.2. Chemotaxis of *G. pallida* towards root exudates of *S. tuberosum* L. cv. Desiree

The wells used in the chemotaxis assays were the source of stimuli with a concentration gradient established around it by the time *G. pallida* J2s were added to the centre of the plate. The results showed that potato root diffusate (PRD) that is routinely used for inducing hatch of PCN J2s did not attract *G. pallida* J2s. Different concentrations of PRD were tested: two-fold concentrated,

* See Glossary for definition.

undiluted and two-fold and four-fold diluted, but the results were the same, that is all were neutral and did not attract the nematodes (Figure 3.13). This indicates that hatching factors and soluble compounds present in PRD are not involved in attraction of J2s of *G. pallida* to potato roots, *i.e.* they do not induce taxis. Devine & Jones (2003) suggested that there was no direct involvement of the hatching factors in host location for *G. rostochiensis* and *G. pallida*. This is in line with the results of the choice (attraction) assays (section 3.1.2) where wheat, which does not exude a HF for PCN, strongly attracted *G. pallida* J2s. A lack of attraction of PRD, even though the roots of *S. tuberosum* Desiree were shown to attract *G. pallida* J2s strongly in section 3.1, indicates that vital compounds for attraction are missing in PRD compared with the living plant. The PRD used for this assay, run-off from a pot of *S. tuberosum* L. cv. Desiree, was stored at -20 °C, which might have contributed to modification of volatile compounds. It is possible that *G. pallida* J2s respond to gaseous (*i.e.* volatile) compounds from host plants (or any plants). It has been shown that the free-living soil nematode *Caenorhabditis elegans* were attracted to volatile organic compounds from *Medicago truncatula* Gaertn (Fabaceae) (Horiuchi *et al.*, 2005). With insects, such as orange wheat blossom midge and black bean aphid, it is reported that location of a host is mediated by a blend of commonly occurring plant volatiles and it is postulated that host-specificity is achieved by the particular ratio between the components (Visser, 1986; Birkett *et al.*, 2004; Webster *et al.*, 2008). For *G. pallida* J2s, which need a HF from the host to hatch and were found in this study to be attracted even to a non-host, it is more

likely that they locate their host responding to a blend of commonly occurring plant volatile or gaseous compounds or possibly one of such compounds.

3.3. Motility assays with root exudates of *S. tuberosum* L. cv. Desiree

PRD-treated or water-treated *G. pallida* J2s had the same speed/rate of kineses (undirected reactions) with both PRD-hatched and water-hatched J2s (Figure 3.14). However, for *G. pallida* J2s, if hatched in PRD, the speed of kinesis (orthokinesis*) was faster compared with those hatched in water, which suggests that pre-conditioning is more important than subsequent treatment conditions, *i.e*. PRD pre-conditioning made the J2s more active in kineses (Figure 3.15). It is interesting that how they hatched appears to have more effect on motility than the treatments they received subsequently. As water-hatched J2s represent those that hatch in the absence of the host in the field, it is only reasonable to assume that they have no reason to move faster in terms kinesis or taxis. Whereas when the J2s hatch responding to HFs from the host in the field, it is highly likely that the J2s have received signals from the host roots to move faster in terms of both kinesis and taxis.

Clarke & Hennessy (1984) reported that *G. rostochiensis* J2s (that hatched in PRD) moved and migrated more when treated with PRD and found that those treated with PRD contained less glycogen and total lipid than those

* See Glossary for definition.

that had been treated with water, presumably as a result of greater activity. In their study, however, the nematodes were immersed in the treatments throughout the assay until the 24 h observation time. In our study, the juveniles (*G. pallida*) were in the treatments for 1 h. Clarke & Hennessy (1984) only tested juveniles (*G. rostochiensis*) that hatched in PRD. It has been shown before that *G. rostochiensis* in the presence of PRD has a dramatic change in its surface cuticle lipophilicity (Akhkha *et al.*, 2002). PRD may not only activate PCN J2s for movement (kinesis) but also prepare them for invasion of root tissues by changing their surface cuticle.

Together with the results from the chemotaxis in section 3.2, it is shown that the PRD that is routinely used for *G. pallida* hatching induces the J2s' positive kinesis, but not taxis, unlike a living *S. tuberosum* Desiree, which suggests that different allelochemicals are involved in activating reactions of kinesis and taxis to the J2s.

3.4. Invasion and development of *G. pallida* in *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

It was shown that second-stage juveniles (J2) of *G. pallida* were strongly attracted to the roots of *S. sisymbriifolium* in the two-way choice assays, and therefore the next question was to what extent they would invade its roots. Interestingly significantly more J2s per gram root were observed in *S. sisymbriifolium* than *S. tuberosum* Desiree at every time point (Figure 3.19).

This may be due to the finer texture of the roots of *S. sisymbriifolium*. Yet when it came to development, those J2s that penetrated the roots of *S. sisymbriifolium* failed to follow the usual development seen in *S. tuberosum*, with no development further than J3. These findings are in line with the report by Roberts & Stone (1983), who found J3s but no J4s, females, cysts or adult males of PCN in the roots of *S. sisymbriifolium* and soil from pots. The fact that some J2s developed to J3 in the current study, although very few, indicates that a feeding site (syncytium) was induced. In resistant plants, it is common that although syncytia are initiated by PCN, there is subsequent incompatibility (Hoopes *et al.*, 1978; Rice *et al.*, 1987; Bleve-Zacheo *et al.*, 1990; Sobczak *et al.*, 2005). In many incompatible* interactions hypersensitive reaction (HR), typically necrosis, is triggered (Hoopes *et al.*, 1978; Rice *et al.*, 1987; Bleve-Zacheo *et al.*, 1990; Sobczak *et al.*, 2005), although it is not necessarily always the case (Golinowski *et al.*, 1997). In the roots of *S. sisymbriifolium* necrosis was not found around the J2s. At 7 wpi, a necrosis-like black area was seen in the J3's head region (Figure 3.21b). Necrosis around the syncytia associated to males has been reported with a beet cyst nematode, *Heterodera schachtii*, on a susceptible *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae), but necrosis seems to appear after males reach the adult stage to leave the root (Sobczak *et al.*, 1997). Although it has not been determined, it is highly likely that the limited number of J3s found in *S. sisymbriifolium* were all male J3. The fact that nematode development was severely hampered suggests that any induced syncytium was so poor that only males, which require much less food than

* See Glossary for definition.

females (Jones & Northcot, 1972), would have been produced. The infective J2s are sexually undifferentiated and sex differentiation becomes visible in the third stage of juvenile, which is thought to be determined by environment, *i.e.* nutritional factors (Trudgill, 1967). With no adult male found, it can be speculated that the J3 could not develop any further, because the syncytium became non-functional and died, hence the blackness around the J3's head region.

Second stage juveniles (J2) of PCN choose an initial syncytial cell (ISC) in the cortex or endodermis and development of syncytium occurs from this ISC towards the vascular cylinder by the formation of a "cortex bridge". This is unique to PCN (Sembdner, 1963; Sobczak & Golinowski, 2009). When the syncytium reaches the vascular cylinder via a cortex bridge, procambial cells next to xylem vessels are incorporated into the syncytium, which spreads along vascular tissues. These procambial cells and also the cortex bridge are hypertrophied. In order for females to develop, incorporation of procambial cells is vital, otherwise the syncytium becomes separated from conductive elements by a layer of compressed cells, which leads to poorer food supply, sufficient to support the development of males only (Sobczak & Golinowski, 2011). Incorporation of procambial cells next to xylem vessels into the syncytium is also crucial for females of another cyst nematode, *Heterodera schachtii,* to develop, although in the case of *H. schachtii* the cell chosen as ISC by the J2 is among procambial or pericyclic cells as opposed to the cortex or endodermis for PCN, and the induction of syncytium in procambial cells rather than in the pericycle is essential for females to develop (Sobczak & Golinowski, 2011).

It is possible that in the interaction between *S. sisymbriifolium* and *G. pallida* J2, procambial cells resist incorporation to the syncytium, hence no females can develop. However, if that had been the only mechanism, adult males should have been observed, and they were not in this study or the study by Roberts & Stone (1983).

It is common to see a shift in sex ratio towards males in incompatible* interactions. For example, between *G. rostochiensis* Ro1 and potato carrying the *H1* resistance gene Forrest *et al*. (1986) reported many males and few females developed on Maris Piper (with *H1* gene), although the total number of adults were fewer than those on a susceptible potato cultivar. Similarly when resistant tomato carrying the *Hero* gene cluster was infected with *G. rostochiensis* Ro1, Sobczak *et al*. (2005) observed many more males develop than females. Van der Vossen *et al*. (2000) also found high numbers of males from potato plants that harboured the *Gpa2* candidate gene *RGC2* when infected with *G. pallida* Pa2, and as for female development Van der Voort *et al.* (1997) reported that some cysts were found on a clone with *Gpa2* locus that was infected with a *G. pallida* Pa2 population.

What seems to be clear from these examples of incompatible interactions is that being “resistant” does not necessarily mean no adult female development. And therefore, the incompatibility between *S. sisymbriifolium* and *G. pallida* appears to be unique, because neither single adult females nor single adult males developed, whilst extremely few J2s developed only to the third stage (J3), which were probably male. It is possible that some toxic compounds

* See Glossary for definition.

are produced by the plant to kill or paralyse the *G. pallida* J2s that invaded the roots. If so these compounds are likely to be produced in response either to the mechanical injuries caused by intracellular migration by the J2s inside the root or to secretions from the J2s via their stylets during migration, or at their attempt to induce syncytia. Another possibility is the involvement of defence-related genes in *S. sisymbriifolium* can lead to immunity to *G. pallida*. It has been shown that various defence genes are down-regulated in feeding sites of nematode-infected susceptible plants; presumably nematode effector compounds are involved in the suppression of the defence response of these plants, possibly to escape host defence mechanisms (Gheysen & Fenoll, 2002; Gheysen & Mitchum, 2009). It is possible that the effector compounds of *G. pallida* fail to induce down-regulation of some of defence genes in *S. sisymbriifolium*. It has also been shown that in the *Hero*- and *Hero A*-gene-mediated incompatible response in tomato to *G. rostochiensis*, *PR-1* (*P4*) was up-regulated (Sobczak *et al.*, 2005; Uehara *et al.*, 2010). Through a systematic study of PR (pathogenesis-related) genes between compatible* and incompatible* plant-nematode systems, the role of critical PR proteins may be uncovered in regulating the success of nematode parasitism (Hamamouch *et al.*, 2011).

In conclusion, the results in this chapter supported only hypothesis iii): "the signalling and behaviour of *G. pallida* in the rhizosphere of *S. sisymbriifolium* is different from that of the host plant *S. tuberosum* L. cv.

* See Glossary for definition.

Desiree, and therefore *G. pallida* J2s i) are not attracted, ii) do not invade , iii) do not develop in *S. sisymbriifolium* roots."

Chapter 4

Novel *in vitro* method to study the development of the potato cyst nematode, *Globodera pallida*

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Introduction

Study of nematode development in any plant of interest is important to assess the compatibility* between the nematode and the plant. This is usually done in pot experiments using soil. However, *in vitro* inoculation of plant roots with infective J2s is more suitable for continuous developmental observations, and usually an axenic medium such as agar is used, but a lengthy process of sterilisation of every material, particularly nematodes, is required.

Pluronic gel has been shown to be useful for studying the behaviour of plant-parasitic nematodes (Wang *et al.*, 2009b; Sasaki-Crawley *et al.*, 2010; Dutta *et al.*, 2011; Reynolds *et al.*, 2011) (also see section 3.1 of Chapter 3). An important characteristic of Pluronic F-127 is that it does not form a rigid gel, unlike agar (Gardener & Jones, 1984), and this allows nematodes to move freely in three dimensions (Wang *et al.*, 2009b). Pluronic F-127 is a non-ionic surfactant, polyoxyethylene-polyoxypropylene-polyoxyethylene (PEOPPO-PEO) triblock co-polymer (Bohorquez *et al.*, 1999), and contains only a low level of substrate impurities (Gardener & Jones, 1984). Thanks to this attribute, Ko & Van Gundy (1988) reported that a hatching experiment with *Meloidogyne incognita* was successfully conducted under non-sterile conditions.

The hypothesis tested in this chapter was that continuous developmental study of *G. pallida* J2s can be successfully conducted in Pluronic F-127 aqueous solution.

* See Glossary for definition.

Materials and methods

Medium

Twenty percent (w/v) of Pluronic F-127 (Sigma-Aldrich) in distilled water including quarter-strength Murashige and Skoog basal salt mixture (Sigma-Aldrich) was placed in a large Duran bottle. The gel was dissolved during sterilisation in an autoclave set at 121°C and kept at 5°C. Plantlets were transferred to Petri dishes containing fresh medium by simply placing them briefly over an ice bath, approximately once a week. This slight decrease in temperature is enough to liquefy the gel allowing the plantlets to be easily lifted without any damage to them.

*Nematodes (*Globodera pallida*)*

See Chapter 2. General Materials and Methods

*Plant materials (*Solanum tuberosum *L. cv. Desiree and* S. sisymbriifolium*)*

See Chapter 2. General Materials and Methods.

Nematode development assays

In a 3.5 cm-diameter Petri dish, *ca.* 150 J2s of *G. pallida* in tap water (5-10 µl) and Pluronic gel (*ca.* 2 ml) were mixed. A *Solanum tuberosum* Desiree or *S. sisymbriifolium* (10- to 14-day-old) plantlet was placed in the Pluronic medium. The Petri dish was then covered with a small piece of aluminium foil with the aerial part of the plantlet exposed. The plantlet was placed inside a magenta box, with tissue saturated with distilled water laid at the bottom of the

box (Figure. 4.1), which was then incubated at 22°C with 16:8 h light:dark. The plantlets were removed from the medium at 19, 20 and 22 days post inoculation (dpi) for *S. tuberosum* Desiree and 19, 20 and 33 dpi for *S. sisymbriifolium*, and the roots were stained with acid fuchsin according to the protocol described by Byrd *et al.* (1983). For *S. tuberosum* Desiree, observation was made at 33 dpi, without staining the roots. Photographs were taken to record nematode development using either a Leica M205FA stereo microscope with Leica Application Suite software or an Olympus inverted microscope with Openlab software.

Figure 4.1. *Solanum sisymbriifolium* at 6 weeks post inoculation with *Globodera pallida* J2s. Scale in cm.

Results

The results proved that continuous developmental study of *G. pallida* J2s can be successfully conducted in Pluronic F-127 aqueous solution under non-sterile conditions. In the roots of *S. tuberosum* Desiree, J2s developed to females (Figure. 4.2a) at every time point (19, 20, 22, 33 dpi), which was repeated in 11 plantlets. Developed females were filled with eggs (Figure. 4.2b) and adult males were also detected. At 33 dpi an adult male was observed mating with an adult female (Figure. 4.2c) in two separate plantlets, which suggests that female pheromones* were properly dispersed in this medium for an adult male to locate an adult female. It demonstrates that this simple system can facilitate observation of *G. pallida* developmental stages in *S. tuberosum* Desiree even until fertilisation.

By contrast, *G. pallida* J2s in *S. sisymbriifolium* showed no further development at every time point in all of the seven plantlets observed even at 33 dpi (Figure. 4.2d), and only one parasitic J2 was found slightly more developed at 20 dpi, which is in agreement with the data obtained from pot experiments (Sasaki-Crawley *et al.*, 2010).

* See Glossary for definition.

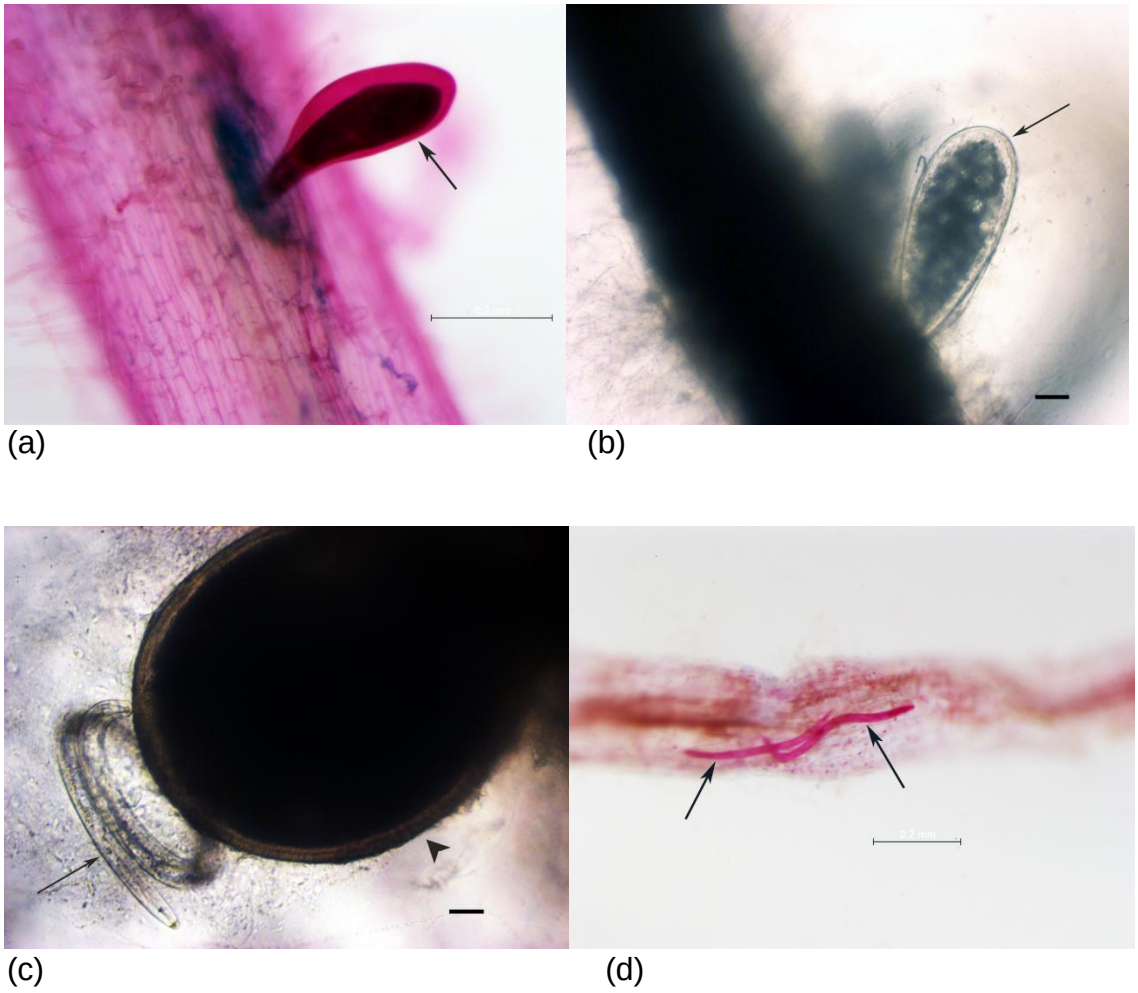


Figure 4.2. (a) Female of *Globodera pallida* (*arrowed*) in the root of *Solanum tuberosum* Desiree at 19 dpi stained with acid fuchsin (scale bar = 0.2 mm), (b) Female of *G. pallida* (*arrow*) filled with eggs in the root of *Solanum tuberosum* Desiree at 19 dpi (scale bar = 50 µm), (c) Mating between an adult male (*arrow*) and an adult female (*arrowhead*) of *G. pallida* in the root of *S. tuberosum* Desiree at 33 dpi (scale bar = 50 µm), (d) Second stage juveniles (*arrow*) of *G. pallida* in the root of *S. sisymbriifolium* at 33 dpi stained with acid fuchsin (scale bar = 0.2 mm).

Discussion

The use of Pluronic gel to observe nematode development has notable advantages over axenic cultures using agar plates. Firstly, surface sterilisation of cysts, J2s or plant material is not necessary. Surface sterilisation of infective stages of plant parasitic nematodes with chemical applications may have an adverse effect on nematodes, such as increased mortality or a possible decline in infecting abilities (Saleh & Fattah, 1990). Antibiotics are often applied, but Forrest *et al*. (1988) reported that cetyltrimethylammonium bromide altered the characteristic structure of the amphidial exudate, thus reducing the ability of *G. rostochiensis* J2s to detect attractants. Heungens *et al.* (1996) described a new rapid method where *ca.* 30 *G. pallida* cysts were sterilised with ethanol and sodium hypochlorite in a 20 ml syringe, but the process becomes much more complicated when a large number of cysts (a scale of several hundred) have to be sterilised.

Secondly, due to its non-rigid texture and transparency, Pluronic gel is an ideal medium for nematode movement in three dimensions, and the infection process is easily observed under a microscope. Also, all roots can be subjected to nematode invasion as opposed to only those exposed on the surface of the agar plates. This affords the potential for more realistic evaluations of nematode-host interactions.

Thirdly, Pluronic gel liquefies when the temperature falls below a certain level for a certain concentration (Gardener & Jones, 1984), which allows the infection process to be easily synchronised. This can be done simply by lowering the temperature of the Pluronic gel and then placing the whole plant

into another container with fresh Pluronic gel without nematodes. This process is harmless to the plant and allows a crude estimation of the number of J2s that have penetrated the roots by counting the number left behind in the original container. Since the medium can be changed as required, the growth of plants can be sustained for unlimited time. Figure 4.1 shows an example with *S. sisymbriifolium*, where the health of the seedling is clearly seen at 6 weeks post inoculation.

The protocol described here has allowed observation of the life-cycle of *G. pallida* in *S. tuberosum* Desiree, and comparative studies were successfully conducted without subjecting the nematode or the seed/plantlet to sterilisation procedures. It could be very useful for screening transgenic or resistant plants or various plant cultivars for their ability to allow the development of plant-parasitic nematodes, and also offers a very useful platform for the collection of different nematode parasitic stages for sequencing or proteomics analysis. The application of this protocol can be extended to the *in vitro* study of the negative/positive interactions of herbivore attack above (insect) and below ground (nematode) on each other through their shared host plant (Wurst & van der Putten, 2007; Kaplan *et al.*, 2008; Hong *et al.*, 2010) or the effect of above and below ground herbivory on the host plant (Kaplan *et al.*, 2008).

Chapter 5

Comparison of defence responses induced by *Globodera pallida* infection in *Solanum tuberosum* L. cv. Desiree and *S. sisymbriifolium*

Introduction

Immunity demonstrated by *S. sisymbriifolium* to *G. pallida* J2 infection in Chapter 3 was unique, as no adult females or males developed. It is possible that i) toxic compounds are produced by the plant to kill or paralyse the nematodes or ii) defence-related genes are up-regulated.

We can speculate that the nematodes are attracted to the roots of *S. sisymbriifolium* and penetrate them, but the plant produces toxic compounds preventing the nematode infection developing further. Plants are known to defend themselves by producing compounds that attack pathogens (Jasmer *et al.*, 2003; Wuyts *et al.*, 2006; 2007). Phytoalexins are one group, this term was originally given by Müller and Borge (1940) and later redefined at a NATO Advanced Study Institute meeting as “low molecular weight, antimicrobial compounds that are both synthesised by and accumulated in plants after exposure to microorganisms” and therefore, the reaction product synthesised by the plant is not specific (Paxton, 1981). Another group comprises reactive oxygen species, which have been shown to be produced in response to nematode invasion (Waetzig *et al.*, 1999; Melillo *et al.*, 2011). In order to test if invaded *G. pallida* J2s are paralysed or killed by toxic compounds produced by *S. sisymbriifolium,* the state of those J2s in the roots of *S. sisymbriifolium* was examined by exposing them mechanically with the help of watchmakers forceps and an insulin needle.

Another possibility to explain the immunity of *S. sisymbriifolium* to *G. pallida* infection may be the result of up-regulation of defence-related genes in *S. sisymbriifolium* that encode pathogenesis-related (PR) and other proteins. PR

proteins, although their discovery was initially associated with hypersensitive response in tobacco leaves to tobacco mosaic virus (TMV) (van Loon & van Kammen, 1970; Gianinaz *et al.*, 1970), were later shown to occur in many other plants that were attacked by other pathogens, such as fungi, bacteria, nematodes and insects (van Loon, 1999). In order to test the hypothesis that defence-related genes are up-regulated in the roots at an early stage of infection with *G. pallida* in *S. sisymbriifolium* but not in *S. tuberosum* Desiree, the changes in gene expression in infected roots of both plants were examined during infection with *G. pallida* J2s by quantitative real-time polymerase chain reaction (qRT-PCR). This was the first real-time PCR analysis conducted for defence-related gene expression in the roots of *S. sisymbriifolium* infected with PCN.

Materials and methods

5.1. Do plant compounds of *S. sisymbriifolium* have a direct effect on *G. pallida* J2s: are the nematodes paralysed or killed inside the roots?

Solanum sisymbriifolium plants inoculated with *G. pallida* J2s in the way described in section 3.4 of Chapter 3 were removed free of soil at 5 dpi. The roots were placed in a 9 cm-diameter Petri dish with water. Without staining the roots, the presence of J2s were estimated from the discolouration of the root (presumably the invasion site). The root cortex was carefully peeled off by using

watchmaker's forceps and an insulin needle under a Wild M5 stereo-microscope to release the J2s from the roots without damaging them. The nematodes freed from the roots of 3 plants were observed under the microscope to determine whether or not they moved.

In order to assess the possibility that *G. pallida* J2s leave the roots of *S. sisymbriifolium* after invasion due to unsuitable conditions, *S. sisymbriifolium* was inoculated with just over 100 *G. pallida* J2s in the method described in Chapter 4 with three replicates. At 4 dpi, the seedling was removed from the gel without damaging the roots by placing the Petri dish over ice briefly to liquefy the Pluronic gel. The roots were gently washed in distilled water to remove any J2s on the surface of the roots. The percentage of the J2s that invaded the roots was estimated by counting the number of the J2s left in the Petri dish plus the ones washed off the root surface. The seedling was transferred to fresh 2 ml of the Pluronic gel including ¼-strength MS in a 3.5 cm-diameter Petri dish and put back to the magenta box system as described in Chapter 4. Thirty five days later, the number of the J2s in the Petri dish was counted to calculate the number that re-emerged from the roots.

5.2. Comparison of defence-related gene expression changes in the time course infection with *G. pallida* between *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*: qRT-PCR analysis

*Nematodes (*Globodera pallida*)*

See Chapter 2. General Materials and Methods.

*Plant materials (*Solanum tuberosum *L. cv. Desiree and* S. sisymbriifolium*)*

See Chapter 2. General Materials and Methods.

Inoculation of plantlets

See Chapter 4, except the number of *G. pallida* J2s for inoculation was just over 500 for a plant. Uninfected plantlets were kept in the same system for a few days before liquid-nitrogen freezing.

RNA extraction

The plantlets of uninfected (control), 3 dpi and 10 dpi with three replicates were frozen with liquid nitrogen and kept at – 80 °C for five to nine months before RNA extraction. The roots were ground in a pestle and a mortar with liquid nitrogen. All the utensils used had been oven baked at 240 °C overnight. The procedure described in the RNeasy Plant Mini Kit Handbook (Qiagen) was followed (2-Mercaptoethanol (Sigma), Ethanol absolute for molecular biology (Sigma)).

cDNA synthesis

The procedure described in the SuperScript™ II Reverse Transcriptase (Invitrogen) was followed (Oligo (dT) 15 Primer (Promega), dNTP Mix (Bioline), RNaseOUT (Invitrogen, Life Technologies Corporation)).

Semi-quantitative PCR to identify genes that are amplified both* in S. sisymbriifolium *and* S. tuberosum *L. cv. Desiree (Primer screening)

Since there is no genomic information about *S. sisymbriifolium,* primers were designed for conserved regions of each gene between potato (*S. tuberosum*) and tomato (*Solanum lycopersicum* (Solanaceae)) for housekeeping and defence-related genes. Twelve primers (Table 5.1), including four housekeeping genes, were tested against uninfected roots of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium* to find the genes that were amplified in both plant species. For each gene, 2 sets of primers of different regions (except for *ACRE*, thaumatin, elongation factor (*EF*), β-tubulin, actin and 18S) were tested to maximise the chance of amplification of the gene in *S. sisymbriifolium* and also to be able to select the primers that perform best in qRT-PCR. Primers were designed to amplify a fragment of 90-150bp in size for each gene. All primers had theoretical melting temperatures (Tm) of *ca.* 60°C and were designed using Primer3 (Untergasser *et al.*, 2007).

For this primer-screening purpose, roots of *ca.* 5 weeks-old plants of both plant species were used, which were grown in 6 x 6 cm multi-cell trays with weed mix as described in section 3.4 of Chapter 3, frozen in liquid nitrogen and kept at – 80 °C (for 6 weeks). For RNA extraction and cDNA synthesis, see

above. For semi-quantitative PCR, first the following were mixed for each primer in a nuclease-free PCR tube: 4 µl of 5x Green GoTaq® Reaction Buffer (Promega), 1 µl dNTP Mix (10 mM each), 1 µl forward primer (10µM), 1 µl reverse primer (10µM), 0.2 µl Go Taq® DNA Plymerase (5 u/µl) (Promega), 2 µl cDNA, 10.80 µl nuclease-free water (Promega) to 20 µl in total. The thermal cycling conditions used were: the initial denaturation at 95 °C for 4 min, 35 cycles of annealing at 95 °C, 57 °C and 72 °C for 30 sec each, the final extension at 72 °C for 5 min, and the soak at 4 °C for an indefinite period of time (C1000 Thermal Cycler, Bio-Rad). The PCR products were separated by agarose gel electrophoresis and visualised with ethidium bromide (200 ml of Tris-acetate-EDTA, 3 g of agarose (Bioline) and 3 µl of ethidium bromide (10 mg/ml, Sigma)). Tris-acetate-EDTA was prepared as below: 50x stock solution was made with 242 g Trizma base (Sigma), 57.1 ml acetic acid (Fluka Chemie GmbH), 100 ml 0.5 M EDTA (Fisher Scientific, pH 8.0) and reverse osmosis water to make 1 L. The working solution was diluted 50 times with reverse osmosis water. The electrophoresis was run at 95 V, 150 mA, 50 W for 20 min.

The semi-quantitative PCR revealed that nine out of 12 genes were amplified in both *S. sisymbriifolium* and *S. tuberosum* Desiree*,* which were *MYB*, *PAL*, *pin*, *ACRE*, chitinase (*ChtC*), *EF*, 18S, β-tubulin and actin.

Gene		Sequence
ACRE-132	Forward	TGAAGGTGAAAAGGCGAGAC
	Reverse	AGGGTTTCGGCATAAAGGAC
ChtC 2. 1	Forward	ATCACTGGAAGATGGCAACC
	Reverse	GCCACCATTGATGATGTTTG
ChtC 2. 2	Forward	TCGGCAGAACAATGTGGTAG
	Reverse	GGGAGAAGGACCAGAAGGAC
GluB 2.1	Forward	TTCTGTTTATGCTGCGATGG
	Reverse	TCATTTCCCAAACCAGCTTC
GluB 2.2	Forward	AGCCCTGTTACTGGCACATC
	Reverse	GTCTTGTGTGGCACCAAATG
MYB 1	Forward	TGTGCATCGTTGGGAGTTAG
	Reverse	GCCCTAGCAGGAACAAGATG
MYB 2	Forward	TGTGCATCGTTGGGAGTTAG
	Reverse	CCCTAGCAGGAACAAGATGC
PAL 1.1	Forward	TGCAAGAGCTGGTGTGAAAG
	Reverse	AAGAGCACCACCATTTTTGG
PAL 1.2	Forward	GCAAGAGCTGGTGTGAAAGC
	Reverse	AAGAGCACCACCATTTTTGG
pin 1	Forward	CTTGGCACGAAAAGAAAGTG
	Reverse	TTCCTTAGCAAGCTTTGTTGG
pin 2	Forward	GCACGAAAAGAAAGTGATGG
	Reverse	TCCATTGTGACTGGAGAACC
PR1b 1	Forward	GTACCAACCAATGTGCAAGC
	Reverse	AAATGAACCACCATCCGTTG
PR1b 2	Forward	GGTACCAACCAATGTGCAAG
	Reverse	GGTACCAACCAATGTGCAAG
thaumatin	Forward	TTGTCCTTTATGCCGAAACC
	Reverse	TTTCGTGTCTCTGTGGAAGC
actin	Forward	CTCTTCGCCGATACCACTCC
	Reverse	TCACACGGTGGAAGGTTGAG

EF	Forward	ATTGGAAACGGATATGCTCCA
	Reverse	TCCTTACCTGAACGCCTGTCA
18S	Forward	GGGCATTCGTATTTCATAGTCAGAG
	Reverse	CGGTTCTTGATTAATGAAAACATCCT
β-tubulin	Forward	ATGTTCAGGCGCAAGGCTT
	Reverse	TCTGCAACCGGGTCATTCAT

Table 5.1. Primers tested against cDNA synthesised from the RNA extracted from uninfected roots of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*.

qRT-PCR

The cDNA of uninfected roots of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium* that were used for the primer screening was diluted 10 times first with nuclease-free water and then further diluted to 5762 ng in 400 µl in total. The concentrations of DNA were measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). The efficiency of PCR for each primer pair was assessed using a serial dilution over 10^4-range of cDNA.

PCR reactions (20µl) contained 4 µl of cDNA (10ng), 10 µl of SYBR Green Jump Start Taq Ready Mix (Sigma) and 0.25 µM of each primer. Samples were run on a Rotor-Gene 6000 (Corbett Research) using the temperature cycling conditions of: 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds, 57°C (for *S. tuberosum* Desiree) or 59°C (for *S. sisymbriifolium*) for 15 seconds, and 72°C for 20 seconds. A final melt-curve step was included post-PCR (ramping from 72°C-95°C by 1°C every 5 seconds) to check for non-specific amplification.

The two most stably expressed housekeeping genes were selected, which were elongation factor (*EF)* and ribosomal 18S. With five defence-related

genes that were amplified both in *S. tuberosum* Desiree and *S. sisymbriifolium*, one pair of primers (f/r) for each gene was selected with a better performance than the other, which were: *PAL 1.2* (phenylalanine ammonia–lyase), *ChtC 2.1* (potato class I basic chitinase), *pin 2* (proteinase inhibitor) and *MYB 2* (v-myb avian myeloblastosis viral oncogene homolog). (*ACRE-132* (*Avr9/Cf-9* rapidly elicited-132) gene had only one pair of primers from the beginning).

Testing gene expressions of uninfected, 3 dpi and 10 dpi of* S. tuberosum *L. cv. Desiree and* S. sisymbriifolium *with* G. pallida *J2s

For synthesis of cDNA, the extracted RNA of each root sample with three replicates was diluted with nuclease-free water according to their RNA concentrations: no nuclease-free water was added to the RNA with the lowest concentration for each plant species, and the volume of nuclease-free water to add was calculated accordingly so that the concentration of RNA would be equal within the species. The synthesised cDNA were first diluted 10 times with nuclease-free water, and then further diluted to 6390 ng in total 400 µl with nuclease-free water. The concentrations of DNA were measured by NanoDrop Spectrophotometer. Each qRT-PCR experiment consisted of three independent biological replicates for all treatments (uninfected (control), 3 dpi and 10 dpi) for all seven primers: 2 housekeeping genes and 5 defence-related genes, with two technical replicates for each. Technical replication was limited to two replicates, i) as PCR reactions were set up using a liquid handling robot (CAS 1200, Corbett Research) which provided exquisite technical reproducibility and ii) to allow us to employ a sample maximisation strategy (*i.e.* running as many

samples as possible in the same run in order to minimise technical run-to-run variation).

Data were analysed according to $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) by first normalising the gene expressions with a housekeeping gene (18S was selected with its stability), and then calibrating them with the uninfected control. The threshold cycle (*C*t) was corrected for the efficiency of each gene.

Statistical analysis

The equation Normalised Relative Quantity (NRQ) = $\frac{2^{-Ct\ \text{Target gene}}}{2^{-Ct\ 18S}}$

$= 2^{-(Ct\ \text{Target gene} - Ct\ 18S)}$

was transformed to $\log_2(1/(2^{-(Ct\ \text{Target gene} - Ct\ 18S)})) = (Ct\ \text{Target gene} - Ct\ 18S)$ (*i.e.* $\log_2(1/NRQ)$), for ANOVA, to obtain values on the *C*t-scale. The effect of treatment (infection with *G. pallida* J2s) was assessed using one-way ANOVA of (*C*t Target gene – *Ct* 18S) for each plant species separately due to 18S having different *C*t-values across them (*S. tuberosum* Desire and *S. sisymbriifolium*). Following ANOVA, relevant means were compared using the least significant difference (LSD) value at the (P = 0.05) level of significance, calculated from the standard error of the difference (SED) on the residual degrees of freedom (df) from the ANOVA. For details, see 5.2 of Appendix I: Statistical analysis.

Results

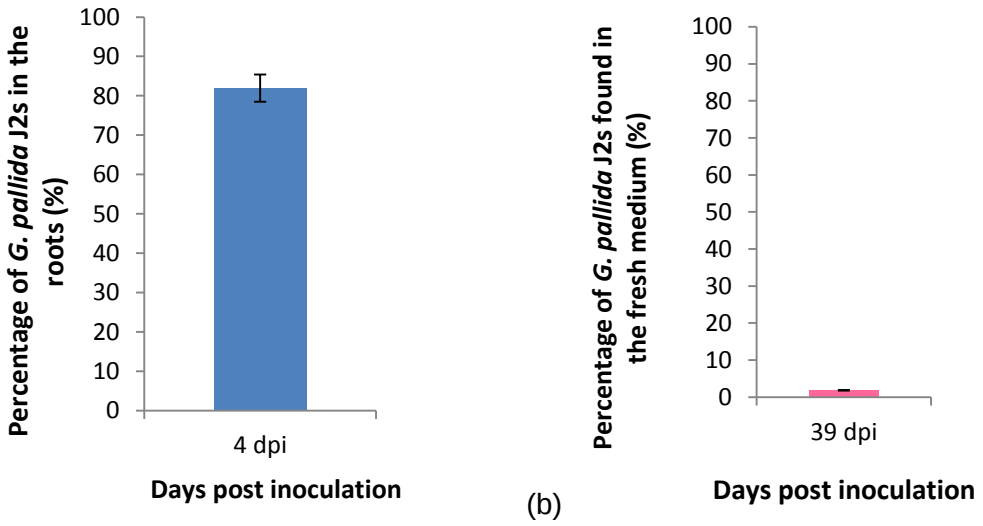
5.1. Do plant compounds of *S. sisymbriifolium* have a direct effect on *G. pallida* J2s: are the nematodes paralysed or killed inside the roots?

A dozen J2s were either totally or partially exposed from the roots of three *S. sisymbriifolium* plants at 5 dpi by using watchmakers forceps and an insulin needle. At least two J2s were clearly moving (Figure 5.1).

Figure 5.1. *Globodera pallida* J2, which was moving after being removed from the root of *Solanum sisymbriifolium* at 5 dpi. (Bar = 10 µm)

The assessment of the possibility that *G. pallida* J2s leave the roots of *S. sisymbriifolium* after invasion due to unsuitable conditions was made with only two replicates as one perished during the experiment, but in both samples, a large percentage of *G. pallida* J2s invaded the roots of *S. sisymbriifolium* with the mean of over 80% (Figure 5.2a). Less than 2% of the total that invaded the roots were found in the fresh medium at 39 dpi (Figure 5.2b). It is more likely that this < 2% J2s had been on the surface of the roots when transferred to the

new medium rather than re-emerged from the roots. If the condition is unsuitable for them, a much higher proportion would have been found outside the roots. And therefore, it would be reasonable to say that the J2s did not leave the roots after the invasion. No females/cysts were observed either on the roots or in the new medium at 39 dpi.



(a) (b)

Figure 5.2. (a) The percentage of *Globodera pallida* J2s that invaded the roots of *Solanum sisymbriifolium* at 4 dpi (n = 2), (b) the percentage of the J2s that were found outside the roots in the fresh medium at 39 dpi (n = 2). Less than 2 % of the J2s that were in the roots at 4 dpi was found in the fresh medium, which indicates that the nematodes did not leave the roots, even though they failed to develop.

5.2. Comparison of defence-related gene expression changes in the time course infection with *G. pallida* between *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*: qRT-PCR analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to compare the expression level of each defence-related gene for *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*. Between two housekeeping genes selected, 18S rather than *EF* was used for normalisation of the real-time PCR data for relative quantification with its more stable *C*t values. Out of five defence-related target genes tested, only the phenylalanine ammonia-lyase (*PAL 1.2*) and the chitinase (*ChtC 2.1*) genes showed difference in gene expression in the time course infection with *G. pallida* J2s in both plant species (Figures 5.3 and 5.4). As three remaining genes, *ACRE-132*, *MYB 2* and *pin 2*, did not show any difference in gene expression over the time course infection, the expression of the *ACRE* gene is shown to represent the *MYB* and the *pin* genes. Gene expression is described in ratio relative to 18S.

Analysing (*C*t Target gene – *Ct* 18S) values, there was no significant effect of treatment (infection with *G. pallida* J2s) for the *ACRE* gene (*ACRE-132*) for both plant species: *P* = 0.507 and 0.239 for *S. tuberosum* Desiree and *S. sisymbriifolium*, respectively; means for treatment were: uninfected 12.382, 3 dpi 12.627, 10 dpi 12.790, SED = 0.3328 on 6 df, LSD (5%) = 0.8143 for *S. tuberosum* Desiree; uninfected 12.842, 3 dpi 12.563, 10 dpi 13.273, SED = 0.3738 on 6 df, LSD (5%) = 0.9147 for *S. sisymbriifolium*). Gene expression did

not change in the time course infection in either plant species (Figures 5.3 and 5.4).

For the *PAL* gene (*PAL 1.2*), there was significant effect of treatment (infection) for both plant species (P = 0.002 and 0.017 for *S. tuberosum* Desiree and *S. sisymbriifolium,* respectively; means for treatment were: uninfected 8.307, 3 dpi 6.210, 10 dpi 5.418, SED = 0.4446 on 6 df, LSD (5%) = 1.088 for *S. tuberosum* Desiree; uninfected 19.15, 3 dpi 16.58, 10 dpi 17.02, SED = 0.662 on 6 df, LSD (5%) = 1.619 for *S. sisymbriifolium*). The *PAL* gene was significantly up-regulated in both plant species at 3 dpi and 10 dpi from uninfected (P < 0.05, LSD) (Figures 5.3 and 5.4). With susceptible *S. tuberosum* Desiree, the expression was further increased at 10 dpi from 3 dpi, but the difference from 3 dpi to 10 dpi was not significant (P > 0.05, LSD), although the increase from uninfected to 10 dpi was significant (P < 0.05, LSD) (Figure 5.3).

With the chitinase gene (*ChtC 2.1*), there was a main effect of treatment (infection) for both plant species (P = 0.025 and 0.009 for *S. tuberosum* Desiree and *S. sisymbriifolium,* respectively; means for treatment were: uninfected 8.458, 3 dpi 7.667, 10 dpi 7.185, SED = 0.3375 on 6 df, LSD (5%) = 0.8258 for *S. tuberosum* Desiree; uninfected 10.207, 3 dpi 8.37, 10 dpi 7.743, SED = 0.5418 on 6 df, LSD (5%) = 1.3258 for *S. sisymbriifolium*). As shown in Figure 5.4, expression of the chitinase gene significantly increased at both 3 dpi and 10 dpi from uninfected with *S. sisymbriifolium* (P < 0.05, LSD), but with susceptible *S. tuberosum* Desiree it was only at 10 dpi, not 3 dpi, when significant up-regulation from uninfected was measured (P < 0.05, LSD) (Figure

5.3), although the magnitude of increase in mean gene expression ratio at 10 dpi was much less (*ca.* one-third) than that with *S. sisymbriifolium* (Figure 5.4).

For statistical details, see 5.2 of Appendix I: Statistical analysis.

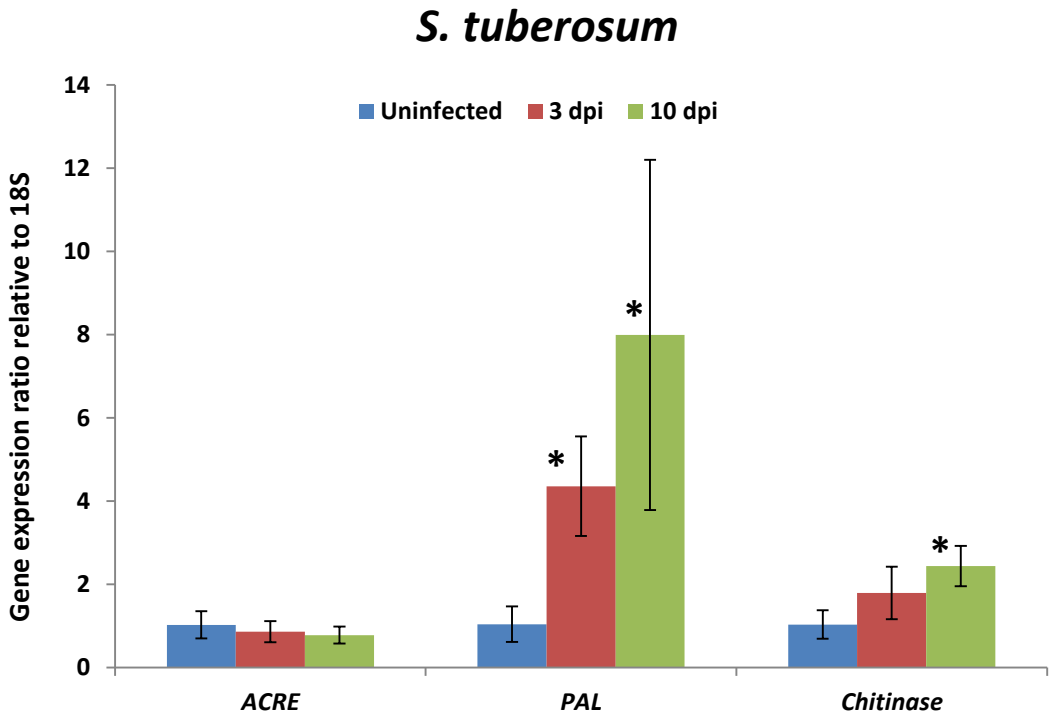


Figure 5.3. The mean (*n* = 3) and 95 % confidence intervals of the gene expression ratio of the *ACRE*, the *PAL* and the chitinase genes relative to 18S in *Solanum tuberosum* Desiree in the time course infection with *Globodera pallida* J2s. Analysing (*C*t Target gene – *Ct* 18S) values, there was no significant effect of treatment for the *ACRE* gene ($P > 0.05$). The *PAL* gene was up-regulated significantly at both 3 dpi and 10 dpi from uninfected ($P < 0.05$, LSD), whereas for the chitinase gene, significant increase of gene expression from uninfected was seen only at 10 dpi ($P < 0.05$, LSD) not at 3 dpi ($P > 0.05$, LSD). Means that are significantly different from uninfected ($P < 0.05$, LSD) are indicated with an asterisk.

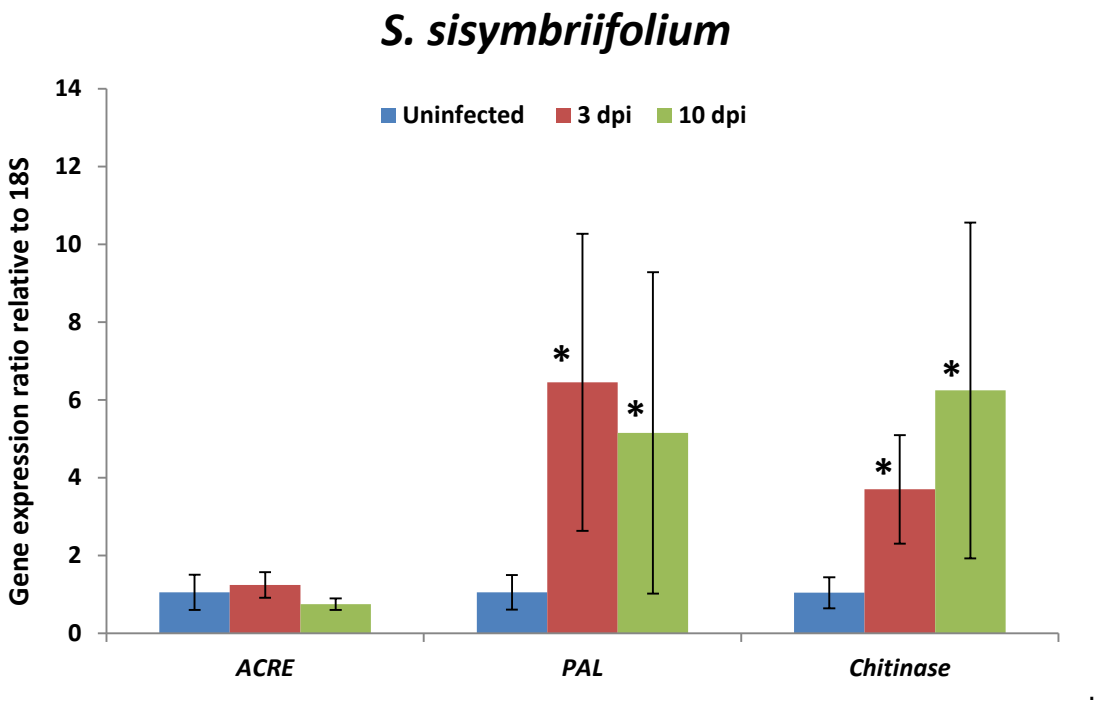


Figure 5.4. The mean (n = 3) and 95 % confidence intervals of the gene expression ratio of the *ACRE*, the *PAL* and the chitinase genes relative to 18S in *Solanum sisymbriifolium* in the time course infection with *Globodera pallida* J2s. Analysing (*C*t Target gene – *Ct* 18S) values, there was no significant effect of treatment for the *ACRE* gene ($P > 0.05$). The *PAL* gene was up-regulated significantly at 3 dpi and 10 dpi from uninfected ($P < 0.05$, LSD), although the expression decreased at 10 dpi from 3 dpi. For the chitinase gene significant increase of gene expression from uninfected was seen at both 3 dpi and 10 dpi ($P < 0.05$, LSD) and the expression increased from 3 dpi to 10 dpi. Means that are significantly different from uninfected ($P < 0.05$, LSD) are indicated with an asterisk.

Discussion

5.1. Do plant compounds of *S. sisymbriifolium* have a direct effect on *G. pallida* J2s: are the nematodes paralysed or killed inside the roots?

With the observation that development of the *G. pallida* J2 that invaded the roots of *S. sisymbriifolium* was impaired, an immediate question was whether or not the plant produces toxic compounds that paralyse or kill the nematodes. The fact that at least two of the J2s that were successfully exposed from the roots of *S. sisymbriifolium* at 5 dpi were clearly moving demonstrated that such compounds are not present in the roots. One could envisage that the toxic compounds may be produced responding to nematodes' penetration of the root, their intracellular migration or their attempt to induce a syncytium. The invasion bioassay results showed that by 3 dpi the number of the nematodes that invaded the roots had reached the peak/plateau, and therefore if the toxic compounds had responded to either penetration or migration, they would have been already paralysed or killed by 5 dpi. As for the possibility that toxic compounds are produced responding to the attempt of inducing a syncytium by J2, Castelli *et al*. (2006) observed the successful induction of syncytia in *S. tuberosum* Desiree at 4 dpi. And therefore, it is reasonable to assume that the attempt of syncytium induction must have been made by 5 dpi in *S. sisymbriifolium*, which suggests that toxic compounds are not involved in the response to the syncytium induction attempt, either.

The possibility that the J2s that invade the roots of *S. sisymbriifolium* subsequently leave the roots due to unsuitable conditions should be eliminated. The observation made in the development assays in section 3.4.2 of Chapter 3 had already supported it with no sign of dramatic reduction of the number of J2s in the roots. However, the result of the pilot experiment using the Pluronic gel described in section 5.1 substantiates this, as less than 2 % of the total J2s that invaded the roots were found outside the roots. If the condition inside the roots of *S. sisymbriifolium* was unsuitable for the *G. pallida* J2s, a much larger percentage should have been found outside the roots.

5.2. Comparison of defence-related gene expression changes in the time course infection with *G. pallida* between *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*: qRT-PCR analysis

If no toxic compounds were involved, or the nematodes do not leave the roots after the invasion, the reason why *G. pallida* J2s do not develop in the roots of *S. sisymbriifolium* may be due to up-regulation of some defence-related genes. This possibility was investigated with eight defence-related genes together with four housekeeping genes: 12 genes in total. Considering there is no genomic information about *S. sisymbriifolium,* it was quite an achievement that nine, including all four housekeeping genes, out of 12 genes were amplified for both *S. sisymbriifolium* and *S. tuberosum* L. cv. Desiree using the primers designed as described in section 5.2 of Materials and methods. The results

revealed a striking contrast between the host *S. tuberosum* Desiree and the trap crop *S. sisymbriifolium* in gene expression with the chitinase gene (*ChtC 2.1*): significant up-regulation at 3 dpi from uninfected in the trap crop (Figure 5.4), but not in the host plant (Figure 5.3). Unlike the chitinase gene, the *PAL* gene (*PAL 1.2*) was up-regulated at 3 dpi and 10 dpi from uninfected in both *Solanum* species (Figures 5.3 and 5.4), while other defence-related genes were not differentially expressed in the time course infection studied.

PAL

The *PAL* gene (*PAL 1.2*) was significantly up-regulated at 3 dpi and 10 dpi in both plant species, although there was a difference in terms of mean gene expression ratio relative to 18S: the magnitude of increase of this ratio at 3 dpi was larger in the trap crop *S. sisymbriifolium* (Figure 5.4) than in the host *S. tuberosum* Desiree (Figure 5.3), but at 10 dpi the ratio decreased from 3 dpi in the trap crop whereas it increased in the host.

Uehara *et al*. (2010) showed that the transcript level of the *PAL* gene (*PAL 1*, *Arabidopsis thaliana PAL* gene) was up-regulated in a resistant tomato cultivar at 3 dpi with *G. rostochiensis* and, just as in this study, the induction was decreased at 7 dpi. However, they found that in a susceptible tomato cultivar it was down-regulated at 3 dpi and slightly induced at 7 dpi. This contradicts our finding and this discrepancy may be due to the difference i) in *PAL* genes investigated (the *A. thaliana* gene for the authors and the potato gene for this study) or ii) in plant species used as the authors used tomato plants for their study. Although tomato is another *Solanaceous* plant, difference

between tomato and potato has been demonstrated in the example where transgenic potato lines carrying the tomato *Hero A* gene failed to confer resistance to PCN, even though a high level of resistance was shown in the transgenic tomato (Sobczak *et al.*, 2005).

The activity of PAL (phenylalanine ammonia-lyase) protein in potato after inoculation with PCN has been shown by Giebel (1973). The author reported that the activity of PAL was higher in a resistant potato cultivar than in a susceptible one after being infected by *G. rostochiensis*. In their study, PAL activity declined in both susceptible and resistant cultivars in the time course infection from the first time point, which was the equivalent of approximately 3 dpi. In this study, a decrease of gene expression was observed only with *S. sisymbriifolium*. The susceptible cultivar used by the author was Pierwiosnek. With other nematode species Brueske (1980) reported that the activity of PAL was significantly increased in resistant tomato after inoculation with *M. incognita* at 108 h (4.5 days) and subsequently decreased, which is in line with the result of this study with *S. sisymbriifolium*. The author tested only a resistant tomato, not a susceptible cultivar.

The PAL protein catalyses the first step of the phenylpropanoid pathway in plants, which leads first to the formation of 4-coumaroyl-CoA and then to the synthesis of various compounds such as flavonoids, isoflavonoids, coumarins, suberin, lignin and other phenolics used by plants in defence to pathogens and environmental stresses (Hahlbrock & Scheel, 1989). However, the findings from the current study strongly suggest that induction and formation of functional syncytia in the host *S. tuberosum* Desiree is not affected by PAL protein activity,

since development of *G. pallida* J2s was evident at 10 dpi as described in section 3.4.2 of Chapter 3.

Chitinase

The significant up-regulation of the chitinase gene (*ChtC 2.1*, a potato class I basic chitinase) in the trap crop *S. sisymbriifolium* at 3 dpi (Figure 5.4) may be the key for the unique immunity of this trap crop against *G. pallida,* as there was no differential expression in the susceptible host *S. tuberosum* Desiree at this time point (Figure 5.3).

Similarly with another PCN, *G. rostochiensis*, strong transcriptional activation of the chitinase gene (*Chi9*, accession No. Z15140, a tomato class I basic chitinase) was shown in a resistant tomato at 3 dpi, but not in a susceptible tomato cultivar (Uehara *et al.*, 2010).

Chitinases can be divided into different classes. Shinshi *et al.* (1990) proposed three classes of plant chitinases based on differences in amino acid sequences, i) class I chitinases are basic with an N-terminal cysteine-rich domain and a highly conserved main structure, ii) class II chitinases are similar to the class I chitinases for the main structure, but lacking the cysteine-rich domain at the N-terminal end and a short extension at the C-terminal end (Legrand *et al.*, 1987), iii) class III chitinases have conserved sequences that are different from those of the class I and II chitinases. For a fourth class, Collinge *et al*. (1993) proposed class IV chitinases, which resemble class I chitinases with a cysteine-rich domain and a conserved main structure but are smaller proteins. Class I chitinases are localised in the vacuole (Boller & Vogeli,

1984; Mauch & Staehelin, 1989; Neuhaus *et al.*, 1991), whereas class II, III and IV chitinases are usually located extracellularly (Legrand *et al.*, 1987; Metraux *et al.*, 1989; Collinge *et al.*, 1993; van Loon *et al.*, 1994; Hamel *et al.*, 1997).

Higher chitinase activity was reported in a root-knot nematode resistant soybean cultivar compared with a susceptible cultivar from 3 dpi onwards (Qiu *et al.*, 1997). As fewer *Meloidogyne incognita* galls and egg masses were found on the resistant cultivar than on the susceptible, the authors speculated higher activity of chitinase detected in the resistant cultivar could be involved in the resistance of soybean to *M. incognita.*

The evidence that chitinases are induced in the roots of *S. tuberosum* after inoculation with *G. pallida* J2s was shown by Rahimi *et al*. (1998). The authors (1998) reported that roots of susceptible *S. tuberosum* Desiree that were infected with *G. pallida* showed chitinase activity with a molecular mass of approximately 60 kDa, which was absent in the uninfected Desiree. In their study, the resistant cultivar tested was the experimental clone P55/7, which has resistance to *G. pallida* Pa1 only and not to Pa2/3, and it was infected with both pathotypes*. The result showed the presence of two bands with chitinase activity with molecular masses of *ca.* 28 and 60 kDa in both Pa1 and Pa2/3 infected roots, but both bands were missing in uninfected roots. In their study, the measurement of chitinase activity was made at only one time point, which was at 1 to 2 weeks post inoculation. That is an equivalent of the result at 10 dpi in this study, where the expression of the chitinase gene (*ChtC 2.1*, a potato class I basic chitinase) was statistically more than that of the uninfected both in

* See Glossary for definition.

susceptible *S. tuberosum* Desiree (Figure 5.3) and *S. sisymbriifolium* (Figure 5.4), although the mean of the gene expression ratio relative to 18S of *S. sisymbriifolium* was nearly 3 times that of *S. tuberosum* Desiree. The authors inferred that the chitinases localised in potatoes infected with *G. pallida* might belong to class III chitinases, as the antibody bound mainly to the chitinases in the extracellular space in the immunolabelling of sections of infected roots. They, however, conceded that other classes of chitinases also might have been induced in potato roots by *G. pallida* but they were not detected due to the binding specificity of the antibody used.

Chitinases hydrolyse the β-1,4-linkage between *N*-acetylglucosamine residues of chitin. Although chitinases have been found in many species of higher plants (Boller *et al.*, 1983), chitin, the natural substrate for chitinase, has not been found in plants so far (Boller, 1987; 1988). It is a chief component of the cell wall of many fungi (Bartnicki-Garcia, 1968; Wessels, 1993), and chitinases have been shown to inhibit fungal growth (Schlumbaum *et al.*, 1986), and their effect is enhanced when combined with another PR protein β-1,3-glucanases (Mauch *et al.*, 1988).

With nematodes, however, chitin has been so far shown to be a component of the eggshells of *G. rostochiensis* and other nematodes (Clarke *et al.*, 1967; Bird & McClure, 1976), but not thought to be a component of the cuticle (Smant & Jones, 2011) or feeding apparatus of plant-parasitic nematodes (Veronico *et al.*, 2001). Veronico *et al*. (2001) found only one chitin synthase gene in *Meloidogyne artiellia* Franklin, which was in eggs but not in hatched invasive J2s or young females.

Here, one can question whether chitin is necessary to induce chitinases in plants. Firstly, even in healthy plants chitinases are constitutively expressed (Collinge *et al.*, 1993; Regalado *et al.*, 2000) even though chitin is not present in plants. Secondly, chitinase activities are shown to increase not only by biotic stresses, such as infection with *Phytophthora infestans* or treatment with the elicitor in *S. tuberosum* leaves (Kombrink *et al.*, 1988; Beerhues & Kombrink, 1994) and infection with *Fusarium solani* f. sp. *pisi* W.C. Snyder & H.N. Hansen in pea pods (Mauch *et al.*, 1984), but also by abiotic stresses, such as treatment with ethylene, chemicals and wounding (Boller, 1988; Broglie *et al.*, 1989) and Beerhues and Kombrink (1994) confirmed it with potato. Chitinases have been shown to be induced not only by fungi but also by bacteria, virus (Metraux & Boller, 1986), viroid (Garcia-Breijo *et al.*, 1990) and nematodes (Qiu *et al.*, 1997; Rahimi *et al.*, 1998), as well as abiotic stresses. Therefore, it makes sense to interpret the induction of chitinase activities as a component of a general mechanism of response to stresses as suggested by Garcia-Breijo *et al*. (1990). And probably therefore, chitinases are induced also in susceptible plants to some extent, although in much less degree compared with resistant cultivars. This difference in magnitude and the timing of the activities may be an important point.

Special attention should be paid to the strong increase of chitinase gene expression in *S. sisymbriifolium* but not in susceptible *S. tuberosum* Desiree at 3 dpi with *G. pallida* (Figures 5.3 and 5.4), as this is the crucial timing for the induction of syncytia: Castelli *et al*. (2006) observed the successful induction of syncytia in *S. tuberosum* Desiree at 4 dpi with *G. pallida*. Strong transcriptional

activation of the chitinase gene (*Chi9,* a tomato class I basic chitinase) was also found in a resistant tomato but not in a susceptible tomato cultivar at 3 days after inoculation with *G. rostochiensis* in the study by Uehara *et al*. (2010). Up-regulation of a chitinase gene at an early stage of infection may determine susceptibility or resistance of the plant to the nematode infection.

Is up-regulation of the chitinase gene (*ChtC 2.1*, a potato class I basic chitinase) related to the failure of *G. pallida* J2s to induce functional feeding sites, syncytia, in *S. sisymbriifolium*? In order for invaded *G. pallida* J2s to develop to females, successful induction of functional syncytia is imperative.

Auxin has been reported to play a crucial part in feeding cell induction by the cyst nematodes *G. rostochiensis* and *Heterodera schachtii* and implicated that its concentration is increased locally by the nematodes (Goverse *et al.*, 2000). The authors point out the importance of polar auxin transport and hypothesise that cyst nematodes achieve local accumulation of auxin at the initial syncytial cell by blocking auxin efflux carriers. Grunewald *et al*. (2009) analysed the role of pin-formed (PIN) auxin efflux carriers in the induction and development of syncytia in Arabidopsis by *H. schachtii* and found that the expression of *PIN1* and *PIN7*, which was present in the vasculature, was missing in the early stage of syncytia, initial syncytial cell (ISC). Therefore the authors suggested that *H. schachtii* J2s directly or indirectly down-regulated *PIN1* and *PIN7* so that the efflux of auxin from the ISC is hampered. Their infection studies with *pin1* and *pin7* Arabidopsis mutants revealed PIN1 plays particularly an important role in this process. One can speculate that significant up-regulation of the chitinase gene (*ChtC 2.1*) at an early stage of infection with

G. pallida J2s in *S. sisymbriifolium* may interfere with down-regulation of *PIN1*, probably indirectly, and therefore, accumulation of auxin is inhibited, which leads to the failure of induction of functional feeding sites. Further speculation is that because significant up-regulation of the chitinase gene is maintained at even higher level at 10 dpi in *S. sisymbriifolium* (Figure 5.4), any later attempt by some *G. pallida* J2s to induce a syncytium is totally hindered, and therefore no adult females or males can develop. It is a contrast to the study by Uehara *et al.* (2010), which showed a few cysts of *G. rostochiensis* developed on the resistant tomato cultivar, in which although the transcription level of the chitinase gene (*Chi9,* a tomato class I basic chitinase) strongly increased at 3 dpi, it decreased later to the level of uninfected at 7 dpi.

Understanding of the interaction between *G. pallida* J2s and the trap crop *S. sisymbriifolium* during the nematodes' attempt to induce a functional feeding site would be greatly enhanced if the option of transcriptome analyses were available, as shown in the interaction between *Meloidogyne incognita* or *M. javanica* and Arabidopsis (Jammes *et al.*, 2005; Fuller *et al.*, 2007; Barcala *et al.*, 2010). Barcala *et al* (2010) showed that a chitinase gene was down-regulated at 3 dpi in a feeding site induced by *M. javanica* in Arabidopsis, using global transcriptome analyses.

This study shows for the first time the changes in gene expression of defence-related genes of the trap crop *S. sisymbriifolium* infected with PCN. The data provides a significant insight into the complex mechanism of *G. pallida* infection in the susceptible host *S. tuberosum* Desiree and the immune trap crop *S. sisymbriifolium*.

In conclusion, the results in this chapter supported the hypothesis that defence-related genes are up-regulated in the roots at an early stage of infection with *G. pallida* in *S. sisymbriifolium* but not in *S. tuberosum* Desiree.

Chapter 6

A novel approach to control *G. pallida* by causing “suicide hatch”

Introduction

One of the novel approaches to control PCN could be causing “suicide hatch” when there are no host plants in the field, that is, when other rotation crops than their host or no crops are grown. Through “suicide hatch”, the PCN population is reduced as hatched J2s starve to death without the host. This method is uniquely possible for PCN unlike other plant parasitic nematodes, because of the following reasons: i) PCN host range is very narrow: only potato, tomato and aubergine are cultivated host plants that PCN can parasitise (Turner & Rowe, 2006), ii) unlike other plant parasitic nematodes, for hatching PCN depend almost entirely on root exudates from the host (Perry, 1989; Perry, 1997). It is thought to be possible to cause such “suicide hatch” by applying host root exudates to the field, and Devine and Jones (2000b) have shown that application of HFs (hatching factor) from tomato root exudate achieved an approximately 50 % reduction of *G. rostochiensis* population size in field trials. As HFs are present in only trace amounts in host root exudates, much effort has been made to synthesise solanoeclepin A (HF of potato), ever since it was isolated by Mulder *et al*. (1996). Recently its synthesis has been finally achieved by Tanino *et al*. (2011).

As *S. sisymbriifolium* induces strong hatch of PCN (Scholte, 2000c), the current study focused on the identification of its HF. It was hypothesised that the roots of *S. sisymbriifolium* also exude solanoeclepin A. In order to test this hypothesis, solanoeclepin A was first searched for in the root exudate of *S. tuberosum* L. cv. Desiree to establish a reliable method, and then the search in the root exudate of *S. sisymbriifolium* followed.

As *S. sisymbriifolium* is chopped up and ploughed into the soil as green manure after growth in the field as a routine practice, hatching ability of the aerial part was also examined.

Materials and methods

6.1. Hatching ability of root exudate extracts

6.1.1. Detection of solanoeclepin A in the root exudate extracts from *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

Collection of root exudates

Fifty *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium* plants were grown hydroponically in liquid medium with 16:8 hours light:dark. The temperature was set at 20°C (day) / 18°C (night) for *S. tuberosum* Desiree and at 20°C/ 20°C for *S. sisymbriifolium*. Sprouts were used for *S. tuberosum* Desiree, whereas for *S. sisymbriifolium* seeds were first germinated in weed mix as described in section 2.2.1 of Chapter 2 and transferred to hydroponics at around 10 days old. The liquid medium used was adapted from Mulder *et al*. (1996), consisting of Murashige and Skoog basal salt mixture (Sigma) with Gamborg’s Vitamin solution 1000 × (Sigma) and 0.25 µM IAA (indole-3-acetic acid) (Sigma) at pH < 4.2. Three plants were grown in 150 to 200 ml liquid medium in a light-shielded glass jar (300 ml). The liquid medium was changed every two to three days and collected when the plants were 5-weeks-old.

Solid-phase extraction (SPE)

The liquid medium collected was subjected to gravity filtration (using Whatman No.1 filter paper) to remove particulate matter and solid-phase extraction (SPE), using a C18 SPE column (500 mg, Biotage). The C18 column was conditioned with high purity methanol (2 ml, Rathburn) and washed through with high purity distilled water (2 ml, Rathburn). After extraction of the medium, the trapped analytes were washed further with high purity distilled water (2ml) to remove interferences, and then extracted using high purity methanol (2 ml). Three quarters of total extract material was dried using dehydrated magnesium sulphate ($MgSO_4$) and the solvent removed using a stream of purified nitrogen until dryness to 0.134 g and 0.03 g for *S. tuberosum* Desiree and *S. sisymbriifolium*, respectively. (For *S. sisymbriifolium*, a second collection of liquid medium was made from another batch, which produced dry yield of 0.085 g from the whole extract material).

Electrospray ionisation-mass spectrometry (ESI-MS)

A sample of the residue obtained from SPE and solvent removal above was prepared for ESI-MS analysis by dissolving a small amount in high purity methanol (1 ml, Rathburn). An aliquot of the sample (20 µl) was subjected to ESI-MS analysis (Autospec Ultima, Waters, Manchester, UK) (positive ionization, +4 kV accelerating voltage, mass range = 100–1000 a.m.u.) by injection through an HPLC injection port into a mobile phase (1:1 methanol: water + 1% acetic acid) under the control of an Agilent 1100 LC pump system.

6.1.2. Investigation of a Hatching Factor (HF) of *S. sisymbriifolium*

6.1.2.1. Determining the optimum concentration of the root exudate extracts from *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium* to induce hatching of *G. pallida* J2s

Hatching assays

Four cysts of *G. pallida* were placed in each well of a 96-well plate (Nunc) and hydrated in 150 µl of tap water for two to three days, and then the tap water was replaced with 150 µl of each treatment with three replicates. The 96-well plate was covered with a lid (of a TPP tissue culture plate (12 wells), which created a better sealant than the lid for the Nunc 96-well plate) and also with Parafilm to minimise evaporation of the treatments, and then it was incubated in darkness at *ca*. 18 °C. At 21 days, the number of the hatched J2s in each well was counted and the percentage of the hatched J2s calculated by dividing the number of hatched J2s by the total number of the eggs in the well. The total number of the eggs per well was estimated by: i) crushing four cysts per well in 100 µl distilled water in a 0.5 ml Eppendorf tube, ii) counting the number of unhatched eggs in a 10 µl aliquot subsample, iii) multiplying it by 10 and the number of the hatched J2s added. This method is a modified version of Devine *et al*. (2001).

In order to determine the optimum concentration of the SPE residue of both *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium* to induce hatching of *G. pallida* J2s, the following concentrations were subjected to hatching assays: the original (undiluted) solution of 1 ml by dissolving 1 mg of the residue obtained from the SPE in 6.1.1 in high purity distilled water (Rathburn), and its serial

dilutions: ×10, $\times 10^2$, $\times 10^3$, $\times 10^4$, $\times 10^5$ and $\times 10^6$ made with high purity distilled water, with the liquid medium used for the hydroponics and high purity water as negative controls.

Statistical analysis

The percentage of hatch (on the square root scale) calculated from the hatching assays was analysed by two-way ANOVA to assess the significance of plant species (*S. tuberosum* Desiree and *S. sisymbriifolium*), concentrations of root exudate extracts (SPE residues) and the interaction of these two factors. Following ANOVA, relevant means were compared using the least significant difference (LSD) value at the (P = 0.05) level of significance, calculated from the standard error of the difference (SED) on the residual degrees of freedom (df) from the ANOVA. For details, see 6.1.2.1 of Appendix I: Statistical analysis.

6.1.2.2. Comparison of hatching ability of reversed-phase HPLC fractions from root exudate extracts of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

HPLC conditions

A C18 reversed-phase HPLC column (ACE, 250 mm x 4 mm) was attached to a Shimadzu SCL-10A_{VP} HPLC system with the mobile phase, comprised of water:methanol (95:5), rising to 100 % methanol over 60 min (flow rate = 1ml / min). Detector wavelength was set at 270 and 350 nm.

Reversed-phase HPLC fractionation

Following the results from section 6.1.2.1, a 0.1 mg/ml was prepared and used in hatching assays. Thus, an aliquot (100 µl) of a 1mg/ml aqueous (high purity distilled water) solution of the SPE residue of *S. tuberosum* Desiree and *S. sisymbriifolium* was subjected to HPLC fractionation in the conditions as described above to obtain four fractions (A, B, C and D, comprising 15 min aliquots, *i.e.* A: 0-15, B: 15-30, C: 30-45, D:45-60 min). Samples were concentrated by rotary evaporation, and frozen samples were subjected to freeze-drying. Dried samples were reconstituted in high purity distilled water to the equivalent of 0.1 mg/ml, and subjected to hatching assays together with a 0.1 mg/ml aqueous solution of unfractionated root exudate SPE residue of both *Solanum* species.

Statistical analysis

The percentage of hatch (on the square root scale) calculated from the hatching assays was analysed by two-way ANOVA to assess the significance of plant species (*S. tuberosum* Desiree and *S. sisymbriifolium*), fractions and the interaction of these two factors. Following ANOVA, relevant means were compared using the least significant difference (LSD) value at the (P = 0.05) level of significance, calculated from the standard error of the difference (SED) on the residual degrees of freedom (df) from the ANOVA. For details, see 6.1.2.2 of Appendix I: Statistical analysis.

6.1.2.3. Bioassay-guided reversed-phase HPLC sub-fractionation of root exudate extract from *S. sisymbriifolium*

***Hatching assay with sub-fractions of fraction B from* S. sisymbriifolium root exudate extract**

With *S. sisymbriifolium,* following the result of the hatching assays in section 6.1.2.2, it was decided to sub-fractionate fraction B (*i.e.* the HPLC fraction collected at 15-30 min). In order to make identification of HF possible by nuclear magnetic resonance (NMR) spectroscopy, a second root exudate extract was collected and prepared for sub-fractionation of fraction B, which produced more SPE residue than the first collection.

An aliquot (200 µl) of a 20 mg/ml aqueous (high purity distilled water) solution of the SPE residue was subjected to reversed-phase HPLC fractionation to obtain three sub-fractions (B-1, B-2 and B-3, comprising 5 min aliquots, *i.e.* 15-20, 20-25 and 25-30 min, respectively). This was repeated 10 times. HPLC conditions were as described in section 6.1.2.2, except a Prominence UFLC (Shimadzu) was used instead of SCL-10A$_{VP}$ HPLC system (Shimadzu). Detector wavelength was set at 270 and 350 nm. Samples were concentrated by rotary evaporation, and frozen samples were subjected to freeze-drying. Dried samples were reconstituted in high purity distilled water to the equivalent of 0.1 mg/ml, and subjected to hatching assays together with a 0.1 mg/ml aqueous solution of unfractionated root exudate SPE residue of *S. sisymbriifolium.*

Statistical analysis

The percentage of hatch (on the square root scale) calculated from the hatching assays was analysed by one-way ANOVA to assess the significance of effect of sub-fractions. Following ANOVA, relevant means were compared using the least significant difference (LSD) value at the (P = 0.05) level of significance, calculated from the standard error of the difference (SED) on the residual degrees of freedom (df) from the ANOVA. For details, see "Hatching assay with sub-fractions of fraction B from *S. sisymbriifolium* root exudate extract" in section 6.1.2.3 of Appendix I: Statistical analysis.

Hatching assay with sub-fractions of fraction B1 from* S. sisymbriifolium *root exudate extract

Following the results of the hatching assay above (Hatching assay with sub-fractions of fraction B from *S. sisymbriifolium* root exudate extract), fraction B-1 was frozen at -20°C and then subjected to freeze-drying to give a residue (300 µg). This residue was reconstituted in high purity distilled water to 300 µl, and 250 µl of this was subjected to reversed-phase HPLC fractionation to obtain five fractions (B1-a, B1-b, B1-c, B1-d and B1-e, comprising 1 min aliquots, *i.e.* 15-16, 16-17, 17-18, 18-19, 19-20 min, respectively). Samples were then subjected to hatching assays together with a 0.1 mg/ml aqueous solution of fraction B-1. HPLC conditions and sample preparation process for hatching assays were as described above (Hatching assay with sub-fractions of fraction B from *S. sisymbriifolium* root exudate extract).

Statistical analysis

See above (Hatching assay with sub-fractions of fraction B from *S. sisymbriifolium* root exudate extract). For details, see "Hatching assay with sub-fractions of fraction B1 from *S. sisymbriifolium* root exudate extract" in section 6.1.2.3 of Appendix I: Statistical analysis.

^{1}H NMR analysis

A fraction containing *S. sisymbriifolium* HF was freeze-dried, and ^{1}H NMR data were acquired on the residue (90 µg) using a Bruker Avance 500 MHz NMR spectrometer in D_2O (deuterium oxide).

Electrospray ionisation-mass spectrometry (ESI-MS)

A fraction containing a HF of *S. sisymbriifolium* (freeze-dried after being frozen at -20°C) was subjected to ESI-MS. Analysis of samples (10ul injections) was performed by ESI-MS using a Quattro Ultima Mass Spectrometer (Micromass / Waters Corporation) coupled to an Agilent 1100 HPLC system in both positive and negative mode (Capillary voltage 2.7KV, Cone voltage 40 and 110, mass range 100-1000 a.m.u). Injections were made manually through an HPLC injection port at a flow rate of 0.1ml/min (mobile phase was 1:1 methanol:water, with 0.05% formic acid in positive mode).

6.2. Hatching ability of aerial part extract

6.2.1. Ethanol extract from aerial part of *S. sisymbriifolium*

The extract was provided by Branston Ltd., and no detail was revealed, apart from that the extract was prepared with 20 % ethanol (EtOH). To test hatching ability of the extract, the following treatments were subjected to hatching assays: undiluted extract, and its 5-fold, 10-fold, 50-fold and 100-fold dilutions with tap water (TW), and with the following controls: TW (negative control), four-fold diluted PRD (collected from 8 weeks-old *S. tuberosum* Desiree) with tap water (positive control), 20 % EtOH and its 5-fold, 10-fold, 50-fold and 100-fold dilutions with tap water. The EtOH with corresponding dilutions with tap water to those of the extract were tested to see if ethanol affected hatching stimulation.

The details of hatching assays were as described in section 6.1.2.1, but three cysts per well instead of four, and also a gas permeable adhesive seal (Thermo) instead of a lid, were used, and observation was made at 7 days, instead of 21 days, to see if any hatching activities were displayed at all.

Statistical analysis

The number of hatched J2s (on the square root scale to account for some heterogeneity of variance) was analysed by general analysis of variance to assess the significance of treatment, concentration and the interaction of these two factors, nesting out the effects of the two control treatments (TW and PRD). Following ANOVA, relevant means were compared using the least significant difference (LSD) value at the (P = 0.05) level of significance,

calculated from the standard error of the difference (SED) on the residual degrees of freedom (df) from the ANOVA. For details, see 6.2.1 of Appendix I: Statistical analysis.

6.2.2. Aqueous extract from macerated aerial part of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

Extract preparation

Solanum tuberosum Desiree and *S. sisymbriifolium* were first grown as described in Chapter 2 and then transferred to 6 x 6 cm cells with weed mix (20 °C (day) / 20 °C (night), 16:8 hours of light:dark). At approximately 10 weeks old, the aerial part was cut into small pieces with scissors and soaked in 100 ml distilled water. The fresh weight of the aerial part used was approximately 10 g for both *Solanum* species. At 72 h, the extract was put through a sieve, and the supernatant of the extract was subjected to hatching assays as described in 6.1.2.1 in the following dilutions with controls: undiluted and its 5-, 10-, 50- and 100-fold dilutions with distilled water, PRD (collected from 7-week-old *S. tuberosum* Desire: 4-fold dilution with distilled water) and distilled water (DW) as positive and negative control, respectively, with three replicates for each treatment.

Statistical analysis

The percentage of hatch (on the square root scale to account for some heterogeneity of variance) calculated from the hatching assay was analysed by

general analysis of variance to assess the significance of plant species, dose, and the interaction of these two factors, nesting out the effects of the two control treatments (DW and PRD). Following ANOVA, relevant means were compared using the least significant difference (LSD) value at the (P = 0.05) level of significance, calculated from the standard error of the difference (SED) on the residual degrees of freedom (df) from the ANOVA. For details, see 6.2.2 of Appendix I: Statistical analysis.

6.2.3. Aqueous extract from intact aerial part of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

Extract preparation

Solanum tuberosum L. cv. Desiree plants were grown from a piece of tuber with a sprout in a 13 cm-diameter pot with Rothamsted standard compost mix (75% Medium grade (L&P) peat, 12% sterilised loam, 3% medium grade vermiculite, 10% grit (5mm, lime free), 3.5 kg Osmocote (3-4 month per m^3), 0.5 kg PG mix per m^3, lime (3 kg to pH 5.5-6.0) and wetting agent (Vitax Ultrawet at 200 ml per m^3) at 20 °C (day) / 20 °C (night) with 16:8 hours of light:dark. *Solanum sisymbriifolium* were grown as described in section 6.2.2. At approximately 6 weeks old, the aerial part was cut out from the plants and immersed in 900 ml distilled water upside down with the cut surfaces out of the water. The fresh weights of the aerial part used were 51.7 g and 43.1 g for *S. tuberosum* Desiree and *S. sisymbriifolium*, respectively. Forty-eight hours later, the aqueous extract was collected and subjected to freeze-drying after being

frozen at -20°C, which yielded dry weights of 0.535 g and 0.6 g for *S. tuberosum* Desiree and *S. sisymbriifolium,* respectively. Two mg of the dry matter was dissolved in 2 ml distilled water, and its 5-, 10-, 50- and 100-fold dilutions with distilled water were subjected to hatching assays as described in section 6.1.2.1, together with PRD (collected from 7-week-old *S. tuberosum* Desiree: 4-fold dilution with distilled water) and distilled water (DW) as a positive and a negative control, respectively. There were three replicates for each treatment.

HPLC profiling

Freeze-dried extract from each *Solanum* species (10 mg each) was dissolved in high purity distilled water (1 ml), 100 µl of which was then subjected to the reversed-phase HPLC analysis in the conditions as described in section 6.1.2.2.

Electrospray ionisation-mass spectrometry (ESI-MS)

The freeze-dried extract from *S. sisymbriifolium* was subjected to ESI-MS as described in section 6.1.2.3.

^{1}H NMR analysis

The freeze-dried extract from *S. sisymbriifolium* was subjected to NMR analysis as described in section 6.1.2.3.

Statistical analysis

See section 6.2.2. For details, see 6.2.3 of Appendix I: Statistical analysis.

Results

6.1. Hatching ability of root exudate extracts

6.1.1. Detection of solanoeclepin A in the root exudate extracts from *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

ESI-MS analysis of the root exudate extract of 50 *S. tuberosum* L. cv. Desiree plants revealed the presence of an $[M+H]^+$ peak at *m/z* 499.95 (Figure 6.1), confirming the presence of the hatching factor, solanoeclepin A ($C_{27}H_{30}O_9$), whose molecular weight is 498.5 (Mulder *et al.*, 1996) with the expected $[M+H]^+$ of 499.5. This result proved that the method applied was correct to detect solanoeclepin A, and therefore the same method was used for *S. sisymbriifolium.* However, the ESI-MS analysis suggested the absence of solanoeclepin A in its root exudate extract (Figure. 6.2).

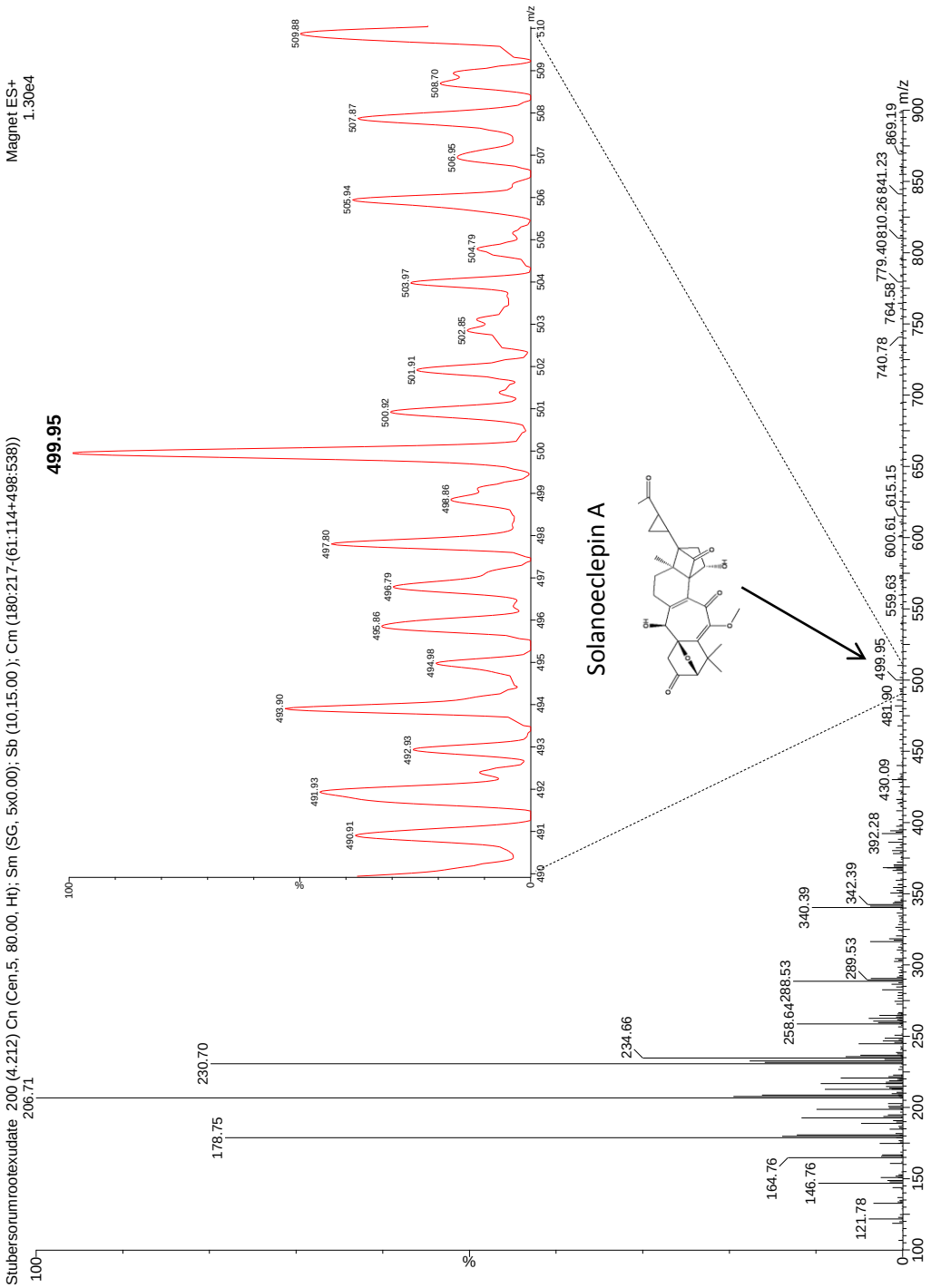


Figure 6.1. Positive ion ESI-MS analysis of *Solanum tuberosum* L. cv. Desiree root exudate extract revealed an $[M+H]^+$ peak at m/z 499.95, confirming the presence of solanoeclepin A ($C_{27}H_{30}O_9$, molecular weight = 498.5 (Mulder *et al.*, 1996), expected $[M+H]^+$ 499.5).

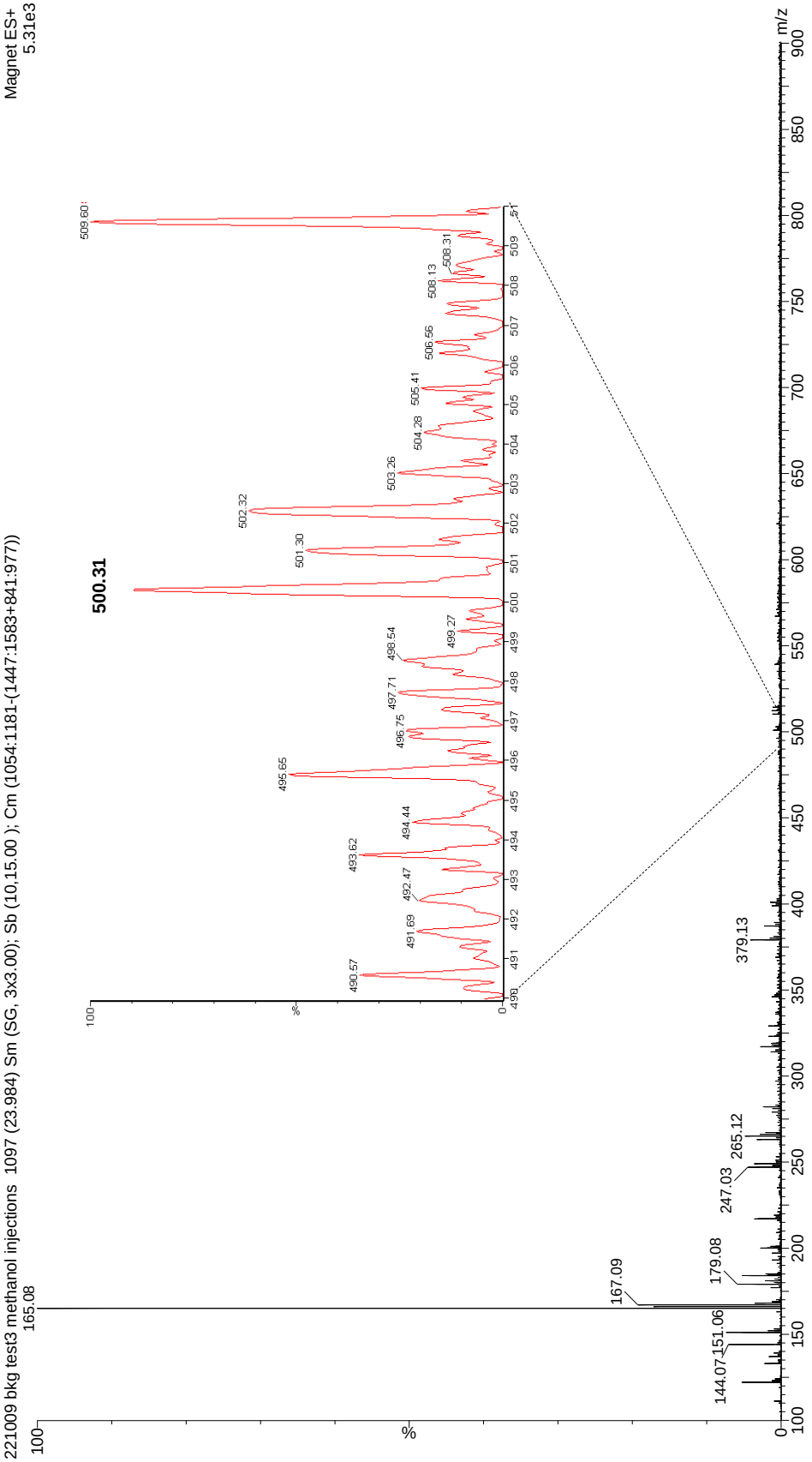


Figure 6.2. Positive ion ESI-MS analysis of *Solanum sisymbriifolium* root exudate extract. There was no peak to confirm the presence of solanoeclepin A ($C_{27}H_{30}O_9$, molecular weight = 498.5 (Mulder *et al.*, 1996), expected $[M+H]^+$ 499.5).

6.1.2. Investigation of a Hatching Factor (HF) of *S. sisymbriifolium*

6.1.2.1. Determining the optimum concentration of the root exudate extracts from *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium* to induce hatching of *G. pallida* J2s

Analysing the percentage of hatched J2s (on the square root scale), there was a main effect of concentration ($P < 0.001$), and evidence of difference between the plant species ($P = 0.050$), but no significant interaction between plant species and concentration ($P > 0.05$). Means were: undiluted 2.654, 10-fold dilution 2.386, 10^2-fold dilution 1.312, 10^3-fold dilution 1.072, 10^4-fold dilution 1.113, 10^5-fold dilution 0.648, 10^6-fold dilution 0.573 (SED for comparisons: 0.1796 on 28 df, LSD (5%) = 0.3679). Means for plant species were *S. tuberosum* Desiree 1.492 and *S. sisymbriifolium* 1.296 (SED for comparison: 0.0960 on 28 df, LSD (5 %) = 0.1967).

For both *Solanum* species, the undiluted aqueous solution of the root exudate extracts induced the highest hatching ability, but not significantly different ($P > 0.05$, LSD) from the 10-fold dilution with high purity distilled water (Figure 6.3). Therefore, it was decided that a 10-fold dilution would be a sufficient concentration for hatching assays. For statistical details, see 6.1.2.1 of Appendix I: Statistical analysis.

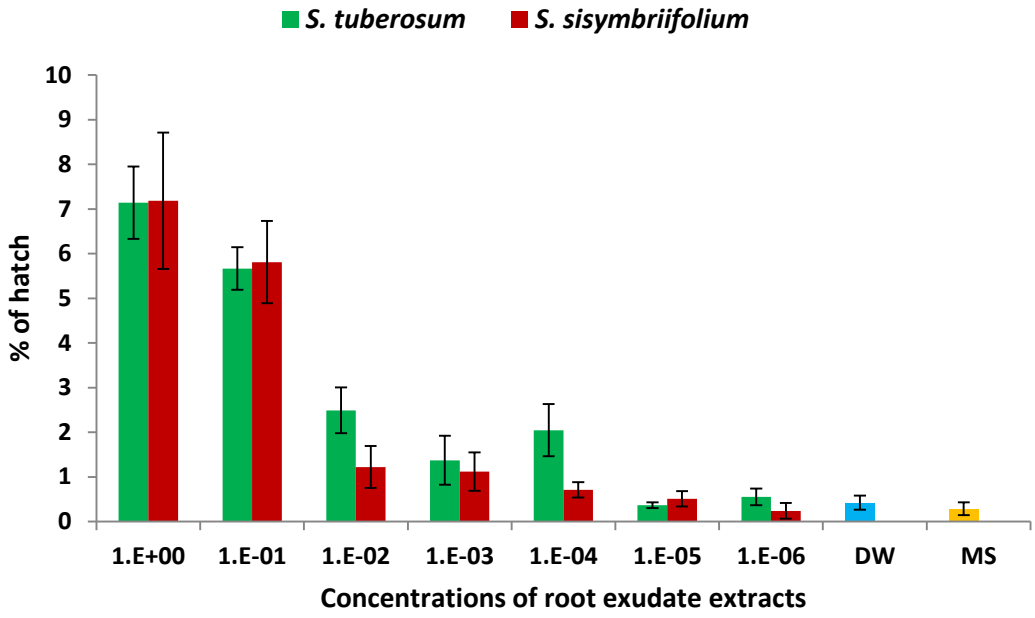


Figure 6.3. The mean (n = 3) and standard error of the percentage of *Globodera pallida* J2s that hatched in different concentrations of root exudate extracts from *Solanum tuberosum* Desiree and *S. sisymbriifolium* at 21 days. DW: high purity distilled water. MS: the liquid medium with Murashige & Skoog basal salt mixture.

6.1.2.2. Comparison of hatching ability of reversed-phase HPLC fractions from root exudate extracts of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

The root exudate extracts from both *Solanum* species were subjected to reversed-phase HPLC fractionation (four fractions) and then to hatching assays in order to compare the hatching ability of each fraction. Analysing the percentage of hatch (on the square root scale), there was no strong main effect of either plant species (P = 0.184) or fractions (P = 0.082), but there was a significant interaction between plant species and fractions (P = 0.004) (Figure 6.4). The highest hatching ability was seen in different fractions between the

two *Solanum* species, *i.e.* fraction C for *S. tuberosum* L. cv. Desiree and fraction B for *S. sisymbriifolium,* and the difference with that of the unfractionated root exudate extract was not significant in both plant species ($P > 0.05$, LSD). For statistical details, see 6.1.2.2 of Appendix I: Statistical analysis.

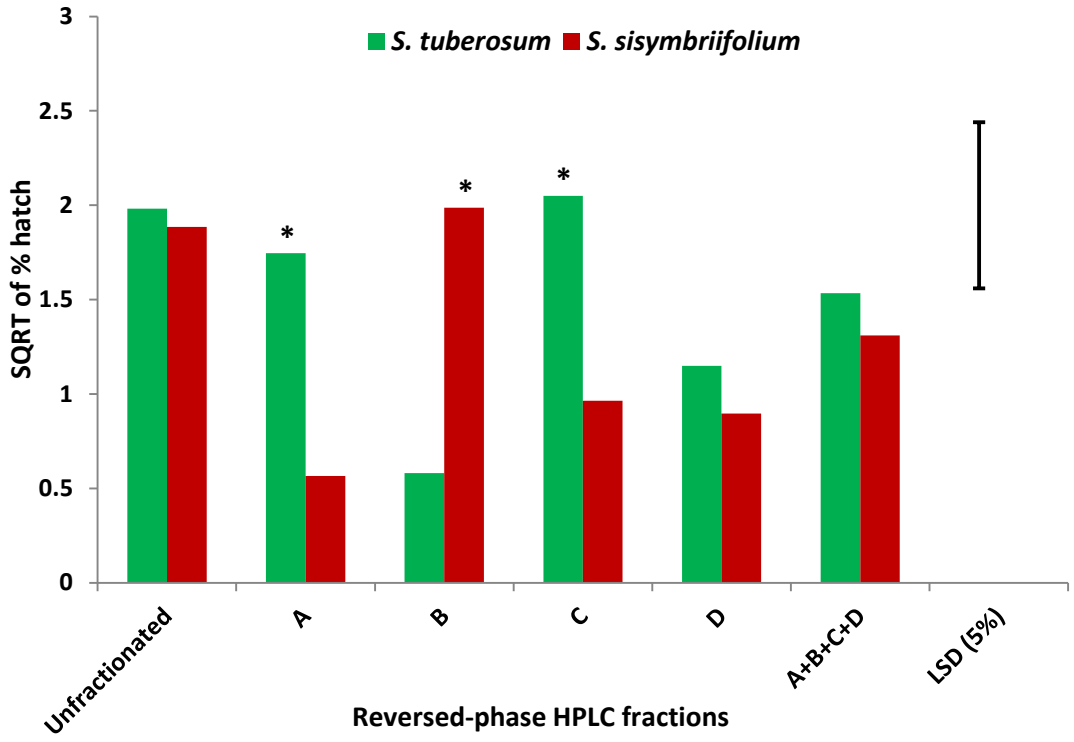


Figure 6.4. The mean square root of the percentage of *Globodera pallida* J2s that hatched in fractions of root exudate extracts from *S. tuberosum* Desire and *S. sisymbriifolium* at 21 days ($n = 3$). The bar represents the LSD (5%). Means that are significantly different ($P < 0.05$, LSD) between plant species are indicated with an asterisk. The highest hatching ability comparable to unfractionated was observed in fractions C and B for *Solanum tuberosum* Desiree and *S. sisymbriifolium*, respectively ($P > 0.05$, LSD).

6.1.2.3. Bioassay-guided reversed-phase HPLC sub-fractionation of root exudate extract from *S. sisymbriifolium*

Hatching assay with sub-fractions of fraction B from* S. sisymbriifolium *root exudate extract

Statistical analysis of % hatch (on the square root scale) showed that there was a significant difference between sub-fractions ($P < 0.001$) (Figure 6.5). Fraction B-1 retained the hatching ability of the unfractionated root exudate extract ($P > 0.05$, LSD), while the hatching ability of B-2 and B-3 was much less and significantly ($P < 0.05$, LSD) different from that of the unfractionated extract. This result suggests fraction B-1 contains a HF. For statistical details, see “Hatching assay with sub-fractions of fraction B from *S. sisymbriifolium* root exudate extract” in section 6.1.2.3 of Appendix I: Statistical analysis.

The HPLC profile shows many activities in fraction B-1. (15 to 20 min) (Figure 6.6).

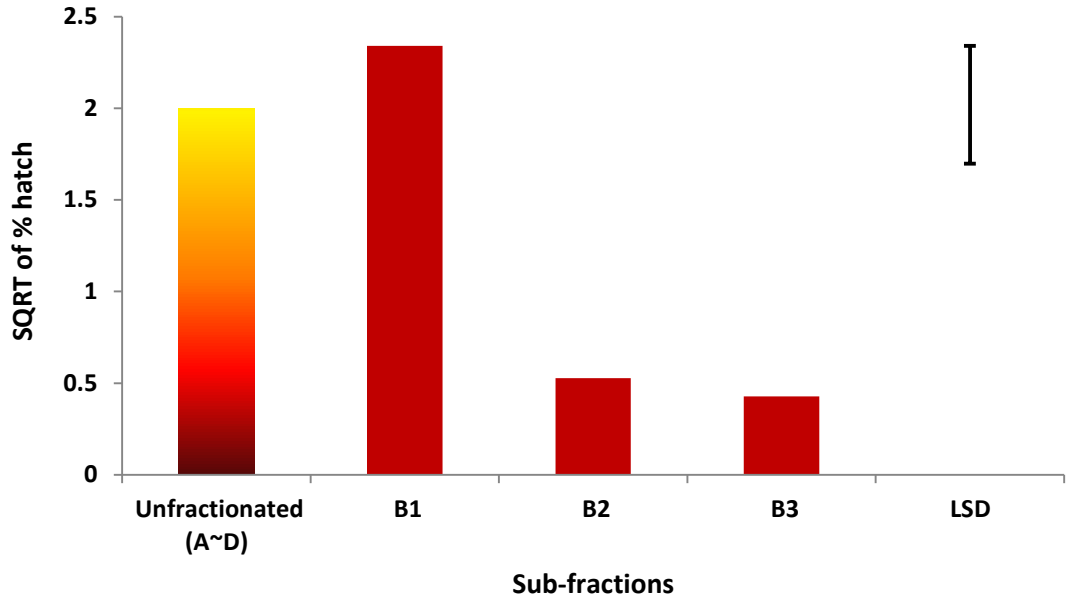


Figure 6.5. The mean square root of the percentage of *Globodera pallida* J2s that hatched in the unfractionated and three sub-fractions of B from *Solanum sisymbriifolium* root exudate extract at 21 days (n = 3). The bar represents the LSD (5 %). The hatching ability of sub-fraction B1 was the highest and comparable to the unfractionated (P > 0.05, LSD).

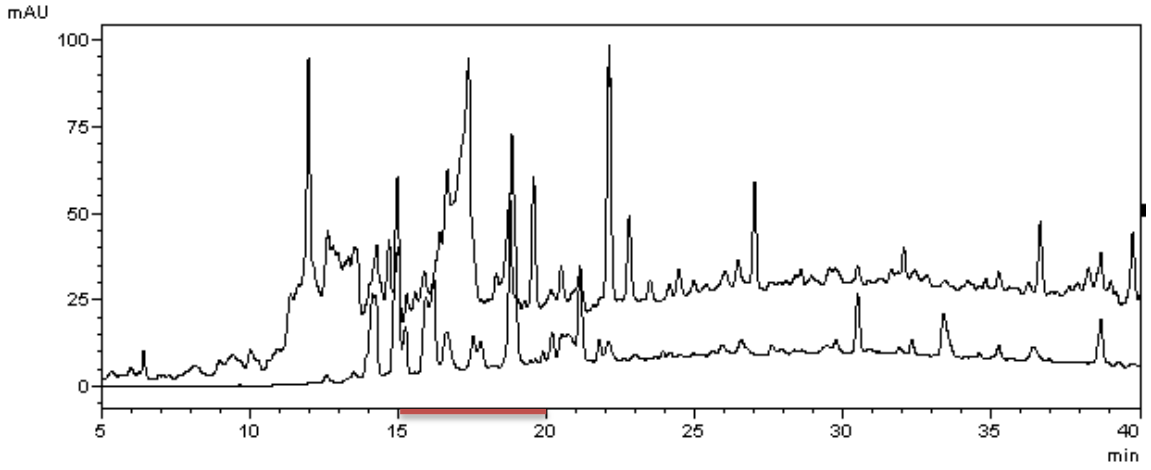


Figure 6.6. HPLC profile for fraction B1 (15-20 min, indicated with red) of *Solanum sisymbriifolium* root exudate extract. The detection wavelengths were 270 nm (upper profile) and 350 nm (lower profile).

Hatching assay with sub-fractions of fraction B1 from* S. sisymbriifolium *root exudate extract

Following the results above (Hatching assay with sub-fractions of fraction B from *S. sisymbriifolium* root exudate extract), the remaining fraction B-1 (15 to 20 min) was subjected to HPLC sub-fractionation to obtain five sub-fractions: B1-a, B1-b, B1-c, B1-d and B1-e (comprising 1 min aliquots) for hatching assays. Statistical analysis of % hatch (on the square root scale) revealed that there was a significant difference between sub-fractions ($P < 0.004$) (Figure 6.7). Fraction B1-d retained the hatching ability of the original B-1 ($P > 0.05$, LSD), and the ability of each of other sub-fractions (B1-a, B1-b, B1-c and B1-e) was significantly different ($P < 0.05$, LSD) from that of B-1. For statistical details, see "Hatching assay with sub-fractions of fraction B1 from *S. sisymbriifolium* root exudate extract" in section 6.1.2.3 of Appendix I: Statistical analysis.

Since the result suggested that fraction B1-d contained a HF, it was subjected to ^{1}H-NMR and ESI-MS (with both positive and negative ionisation) analyses to identify the HF. However, there were no detectable signals to report for either ^{1}H-NMR or ESI-MS analysis.

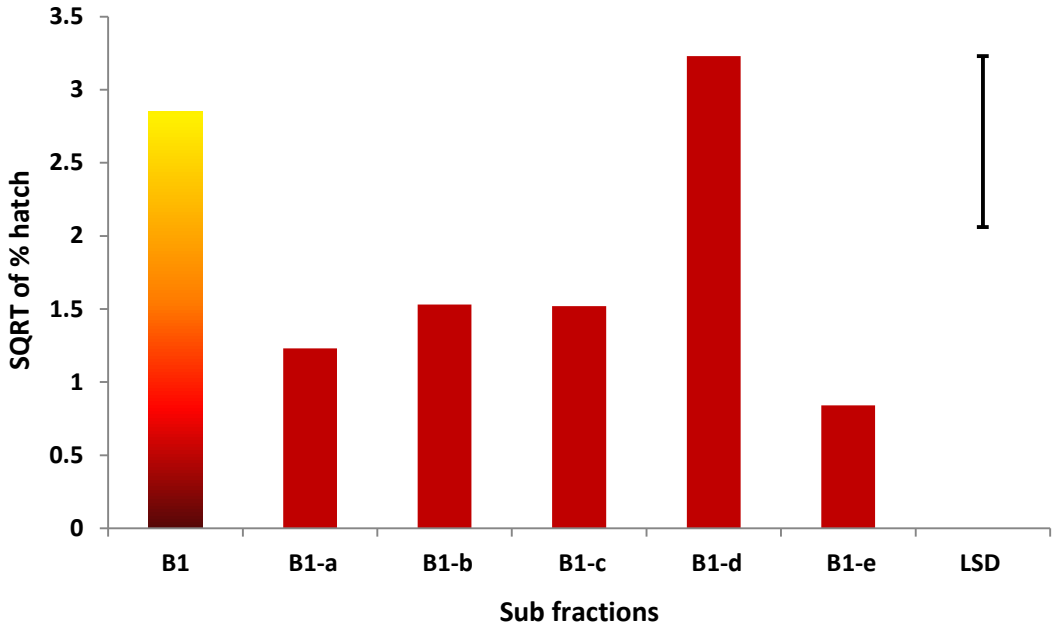


Figure 6.7. The mean square root of the percentage of *Globodera pallida* J2s that hatched in fraction B1 and its five sub-fractions from *Solanum sisymbriifolium* root exudate extract at 21 days (n = 3). The bar represents the LSD (5 %). The hatching ability of sub-fraction B1-d was the highest and comparable to that of B1 (P > 0.05, LSD).

6.2. Hatching ability of aerial part extract

6.2.1. Ethanol extract from aerial part of *S. sisymbriifolium*

Analysing the number of hatched J2s on the square root scale, there was a main effect of concentration of treatment (P = 0.032), but there was no significant difference between treatments (*i.e.* between *S. sisymbriifolium* ethanol extract and ethanol) (P = 0.899) and also no significant interaction between treatment and concentration. Means (square root scale) for controls and concentrations (over both treatments) were: TW 0.33, PRD 9.34, undiluted 1.71, 5-fold dilution 4.42, 10-fold dilution 2.70, 50-fold dilution 2.37, 100-fold dilution 2.19 (SEDs for comparisons: PRD vs. TW 1.166 on 24 df, LSD (5%) = 2.407; PRD or TW vs concentrations 1.010 on 24 df, LSD (5 %) = 2.085; between concentrations 0.825 on 24 df, LSD (5%) = 1.702). The 5-fold dilution was significantly different (P < 0.05, LSD) from the rest, inducing the strongest hatching. Clearly, the 5-fold dilution of the *S. sisymbriifolium* ethanol extract was most responsible for this effect (Figure 6.8). For statistical details, see 6.2.1 of Appendix I: Statistical analysis.

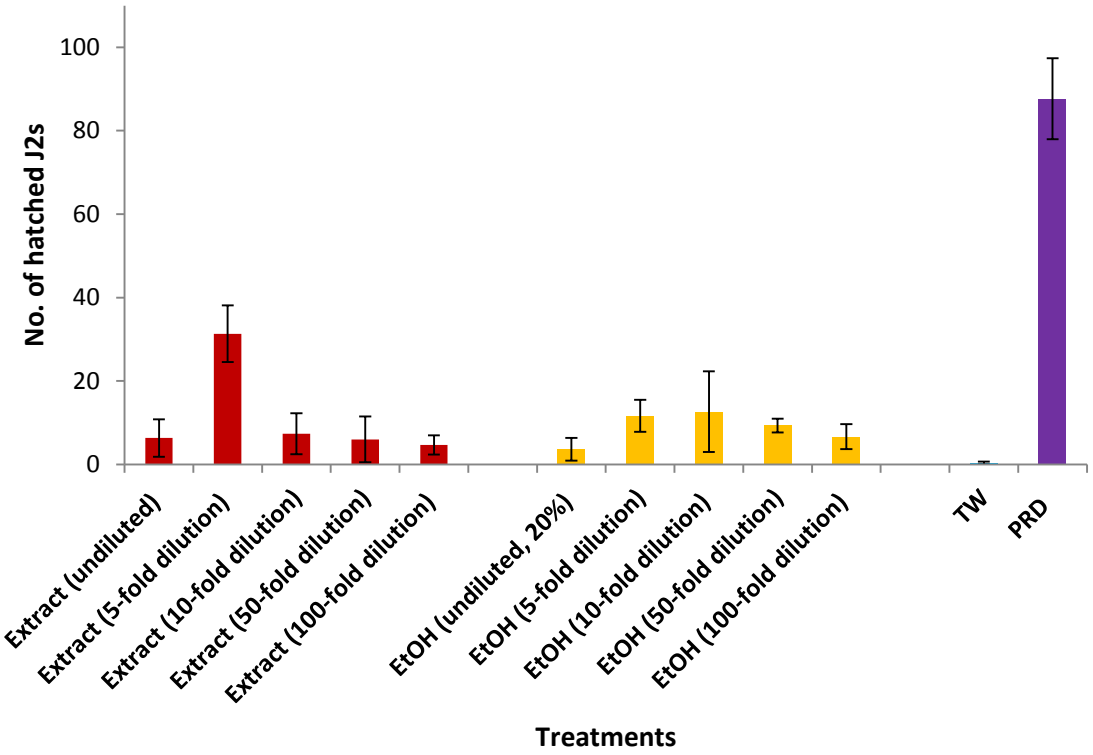


Figure 6.8. The mean (n = 3) and standard error of the number of *Globodera pallida* J2s that hatched in *Solanum sisymbriifolium* ethanol aerial part extract with different concentrations diluted with water and also in ethanol only with corresponding concentrations at 7 days. Five-fold dilution of the *Solanum sisymbriifolium* ethanol aerial part extract induced strong hatching.

6.2.2. Aqueous extract from macerated aerial part of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

Interestingly, the aqueous extract from macerated *S. tuberosum* L. cv. Desiree aerial part showed a fairly good hatching ability at every dose (fresh weight mg/ml) (Figure 6.9). By contrast, the extract from *S. sisymbriifolium* had to be diluted 5 times with distilled water before showing a comparable hatching ability, and its hatching activity peaked at 10-fold dilution and gradually decreased with further dilutions. Analysing the percentage of hatch (on the

square root scale), there was a significant interaction between plant species and dose (P = 0.009), along with a significant main effect of dose (P = 0.008), but not of plant species (P = 0.306). At 100 mg/ml, *S. tuberosum* Desiree induced significantly more hatching than *S. sisymbriifolium* (P < 0.05, LSD), but this was reversed at 10 mg/ml (P < 0.05, LSD). At 10 mg/ml the hatching ability of *S. sisymbriifolium* extract showed statistically no difference from that of PRD (P > 0.05, LSD). With *S. tuberosum* Desiree, however, the hatching activity never rose to a level comparable to that of PRD at any dose (P < 0.05, LSD). For statistical details, see 6.2.2 of Appendix I: Statistical analysis.

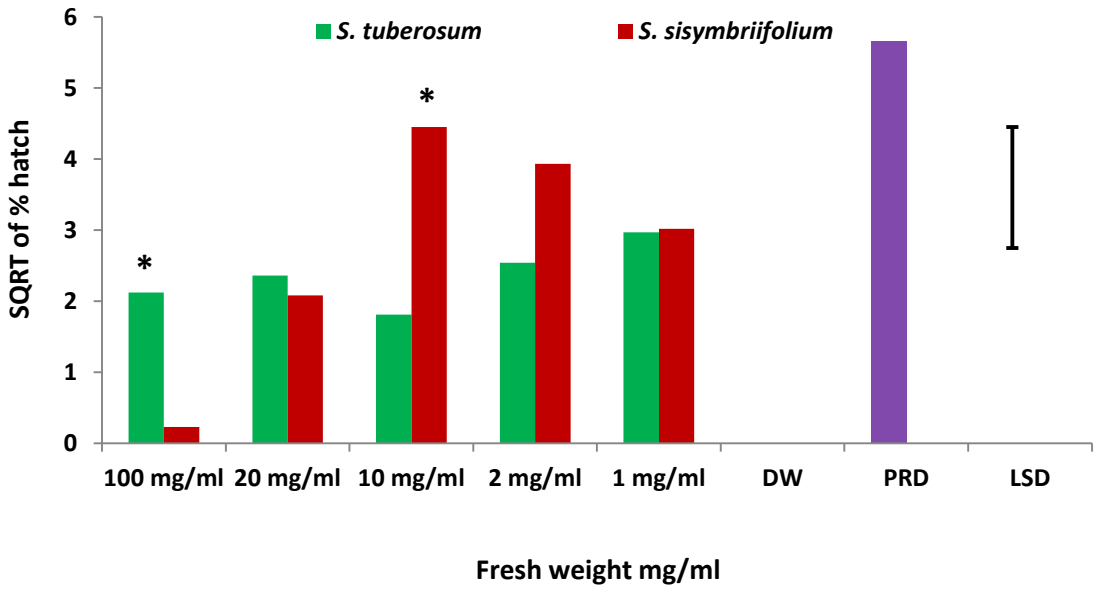


Figure 6.9. The mean square root of the percentage of *Globodera pallida* J2s that hatched in macerated aerial part (aqueous) extract from *Solanum tuberosum* Desiree and *S. sisymbriifolium* at 21 days (n = 3). The bar represents the LSD (5 %). The hatching ability of *S. tuberosum* Desiree extract at 100 mg/ml was significantly more than that of *S. sisymbriifolium* (P < 0.05, LSD), which is indicated with an asterisk, but it was reversed at 10 mg/ml (P < 0.05, LSD). The hatching ability of *S. sisymbriifolium* extract at 10 mg/ml was as strong as that of PRD (P > 0.05, LSD).

6.2.3. Aqueous extract from intact aerial part of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

6.2.3.1. Hatching assay

With intact aerial part aqueous extract, *S. sisymbriifolium* showed a strong hatching ability at the three highest doses (Figure 6.10). Analysing the percentage of hatch (on the square root scale), there was a significant interaction between plant species and dose ($P = 0.016$), along with a significant main effect of plant species ($P < 0.001$) and of dose ($P < 0.001$). At each of the three highest doses the hatching ability of *S. sisymbriifolium* extract showed a significant difference from that of *S. tuberosum* Desiree ($P < 0.05$, LSD), but not from that of PRD ($P > 0.05$, LSD). The three highest doses, 80 m/ml, 16 mg/ml and 8 mg/ml are approximately equivalent to 1 mg/ml, 0.2 mg/ml and 0.1 mg/ml, respectively in terms of dry matter obtained from freeze-drying. The approximate fresh weight dose (mg/ml) was calculated from the original fresh weight of the aerial part used for extract preparation and the dry matter weight yielded from its freeze-drying. The purpose of this calculation was to be able to make an approximate comparison with the hatching ability of the macerated aerial part aqueous extracts. For statistical details, see 6.2.3 of Appendix I: Statistical analysis.

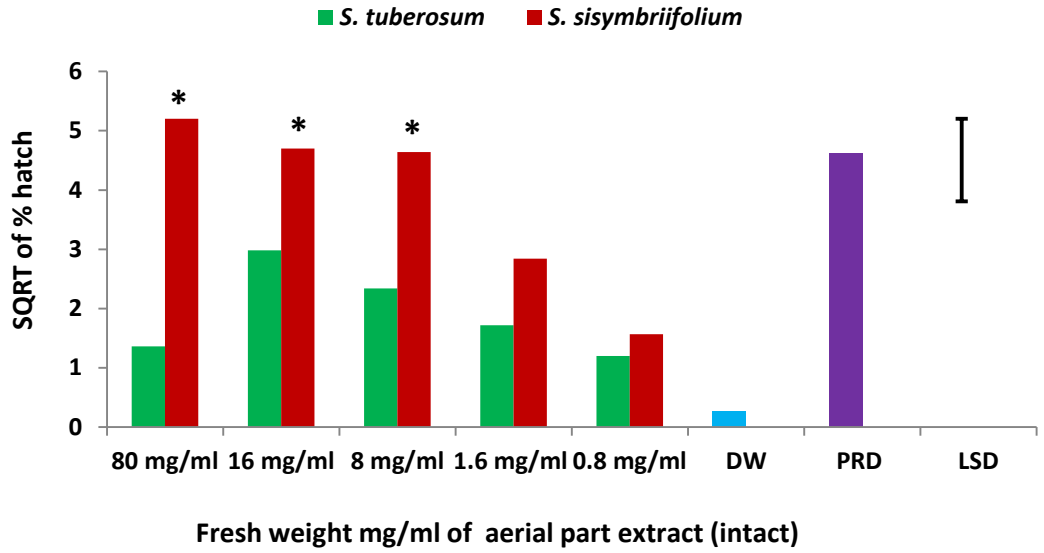
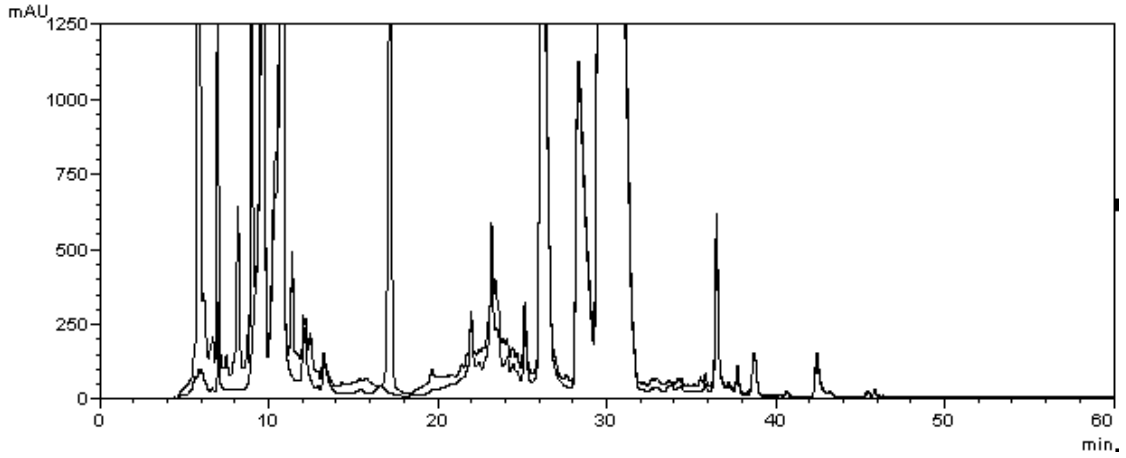


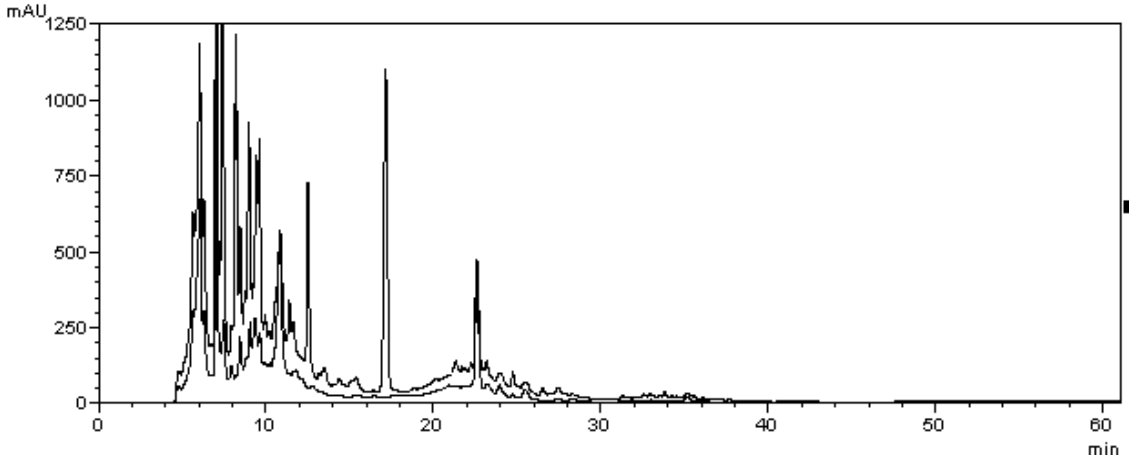
Figure 6.10. The mean square root of the percentage of *Globodera pallida* J2s that hatched in intact aerial part (aqueous) extract from *Solanum tuberosum* Desiree and *S. sisymbriifolium* at 21 days (n = 3). The bar represents the LSD (5 %). The hatching ability of *S. sisymbriifolium* extract at the three highest doses was significantly different from that of *S. tuberosum* Desiree ($P < 0.05$, LSD), which is indicated with an asterisk, but comparable to PRD ($P > 0.05$, LSD).

6.2.3.2. HPLC profiles

The reversed-phase HPLC profile of *S. sisymbriifolium* aqueous extract from its intact aerial part showed much stronger signals throughout (Figure 6.11a) than that of *S. tuberosum* Desiree (Figure 6.11b), particularly from 15 to 30 min. This could suggest the presence of a HF in the retention time between 15 and 30 min, and this finding is in line with the hatching assay results with the root exudate extract of *S. sisymbriifolium* (see section 6.1.2.2).



(a)



(b)

Figure 6.11. Profiles of reversed-phase HPLC for aqueous extract from intact aerial part of: (a) *Solanum sisymbriifolium,* (b) *S. tuberosum* L. cv. Desiree. The detection wavelengths were 270 nm (upper profile) and 350 nm (lower profile).

6.2.3.3. Chemical analysis

^{1}H-NMR analysis of the aqueous extract from intact aerial part of *S. sisymbriifolium* suggests the presence of signals (6.0 ~ 8.0 ppm) relating to

aromatic ring structures, possibly arising due to the presence of phenolic/flavonoid type chemistry (Figure 6.12). The presence of other signals (2.8 ~ 4.5 ppm) strongly suggests the presence of sugar units.

Subsequent analysis of the extract by ESI-MS with negative ionisation (Figure 6.13) provides further evidence for these findings. The presence of ions at 353 and 191 suggests the presence of chlorogenic acid ($C_{16}H_{18}O_9$, molecular weight = 354.31, the ester of caffeic acid with quinic acid (molecular weight 192.17)) and at 595 the presence of a flavonoid diglycoside, such as eriocitrin (molecular weight = 596.54). There appeared to be no detectable sign of solasodine (glycoalkaloid), a previously identified leaf component from *S. sisymbriifolium* (Pandeya *et al.*, 1981; Subramani *et al.*, 1989), although positive ion ESI-MS analysis tentatively suggested the presence of one of the potato glycoalkaloids, solanine ($C_{45}H_{73}NO_{15}$, molecular weight = 868.06) with an $[M+H]^+$ peak at 869.71 as shown in Figure 6.14.

Also, there is no detectable sign of the following identified previously from leaves (aerial material) of members of Solanaceae: glycoalkaloids (solamargine, solasonine) (Tingey & Sinden, 1982), acyl-sugars (6-*O*-acetyl 2,3,4-tri-*O*-[3-methylvaleryl]- α-D-glucopyranosyl-β-D-fructofuranoside, 3,4-di-*O*-isobutyryl-6-*O*-caprylsucrose, 3,4,6-tri-0-, 3,3',4,6-tetra-0- and 2,3,3',4-tetra-0-acylated sucrose ester complex, 6-*O*-capryl-3,3′,4-tri-*O*-isobutyrylsucrose, 6-*O*-capryl-3'-*O*-isobutyryl-3**,**4-di-*O*-(2-methylbutyryl) sucrose**,** 3-di-*O*-hexanoyl-α-glucopyranose, 1,2,3-tri-*O*-hexanoyl-α-glucopyranose and 2,4,1′-tri-*O*-(3-methylbutyryl)-3-*O*-(2-methylbutyryl) sucrose) (Severson *et al.*, 1985; King *et al.*, 1986; King *et al.*, 1987a; King *et al.*, 1987b; King & Calhoun, 1988; King *et al.*,

1990), diterpene glycosides (Keinänen *et al.*, 2001) or other flavonoids (Wollenweber *et al.*, 2005).

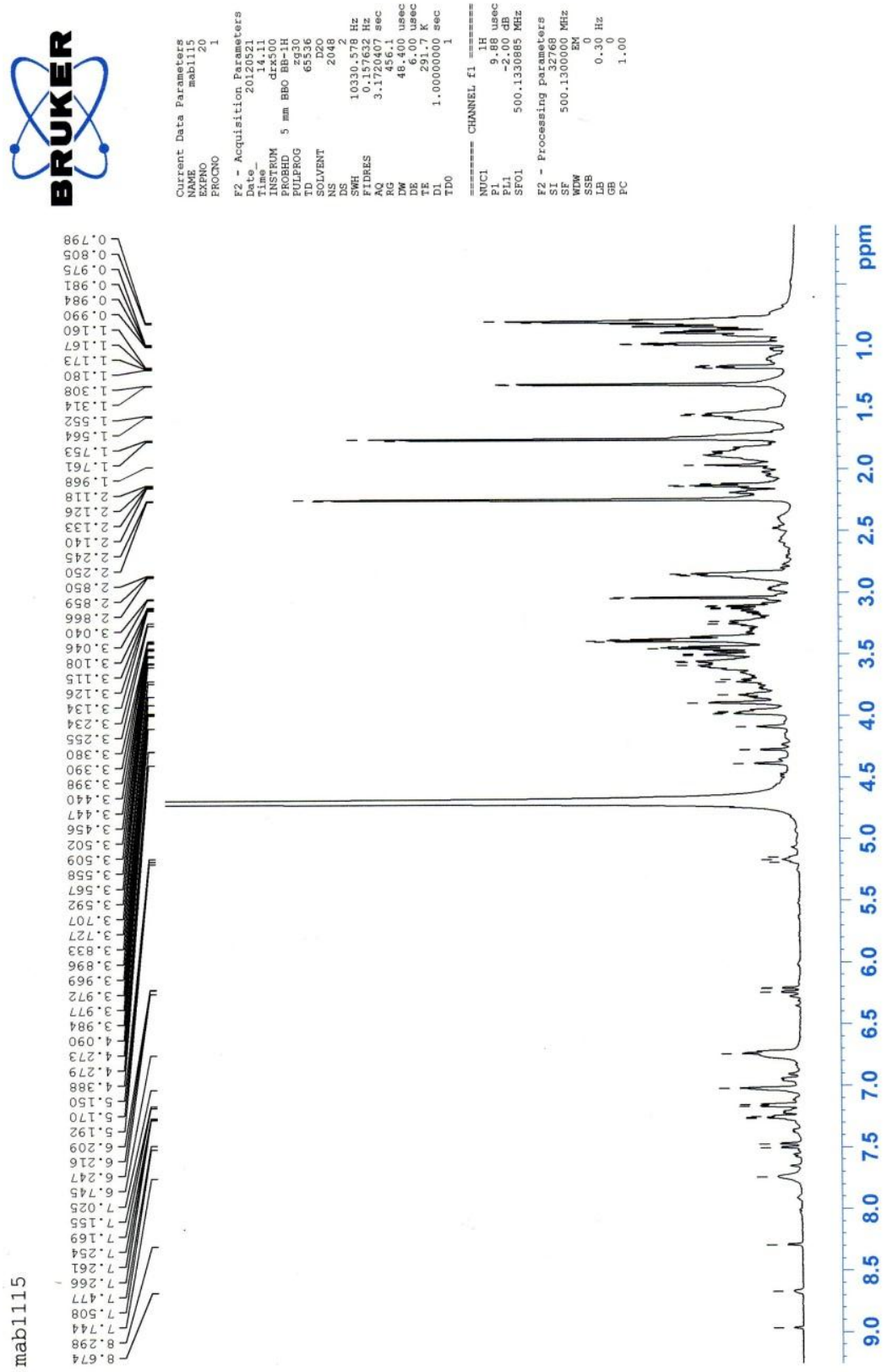


Figure 6.12. ^{1}H NMR spectrum of the aqueous extract from intact aerial part of *Solanum sisymbriifolium*.

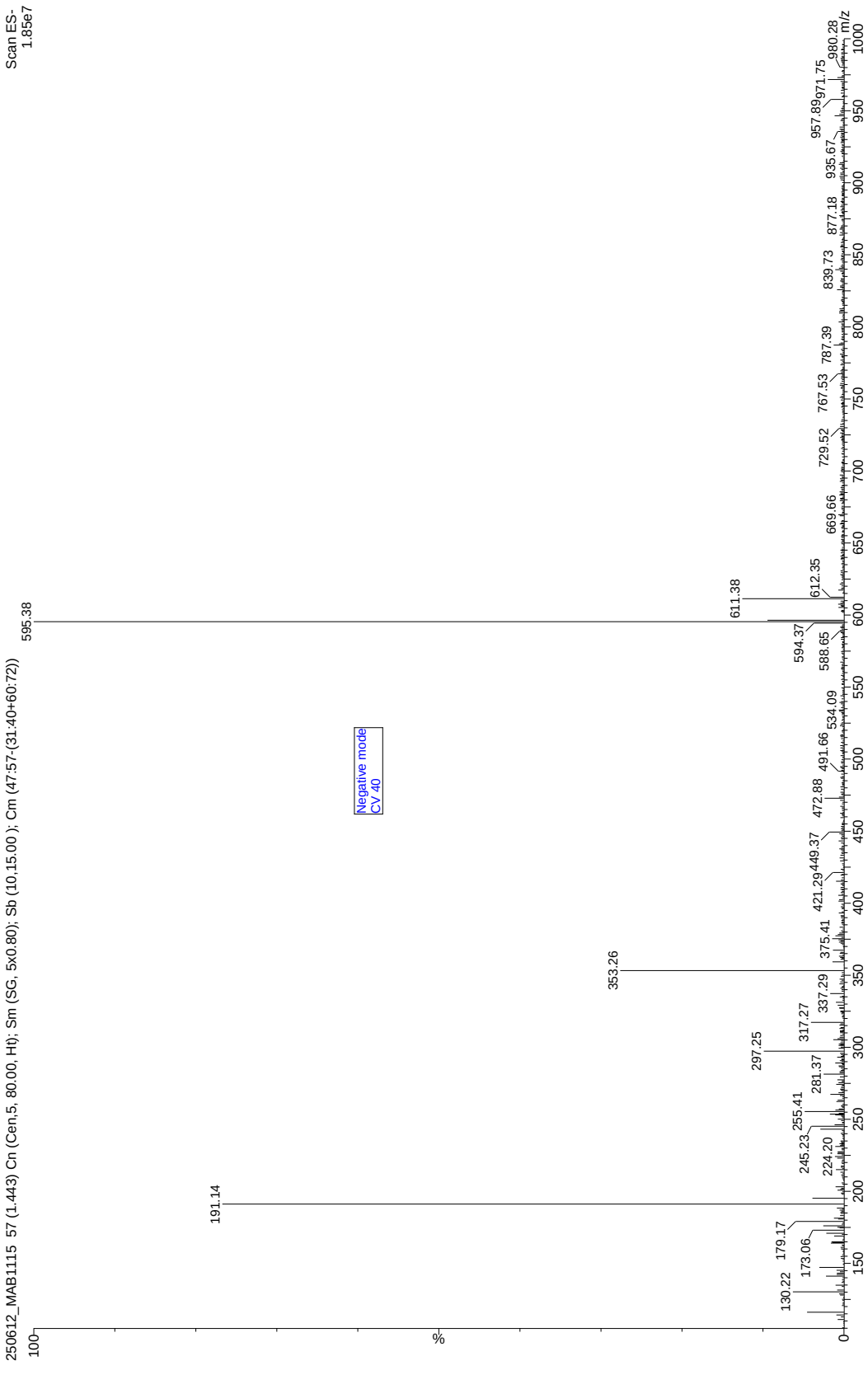


Figure 6.13. Negative ion ESI-MS analysis of the aqueous extract from intact aerial part of *Solanum sisymbriifolium*

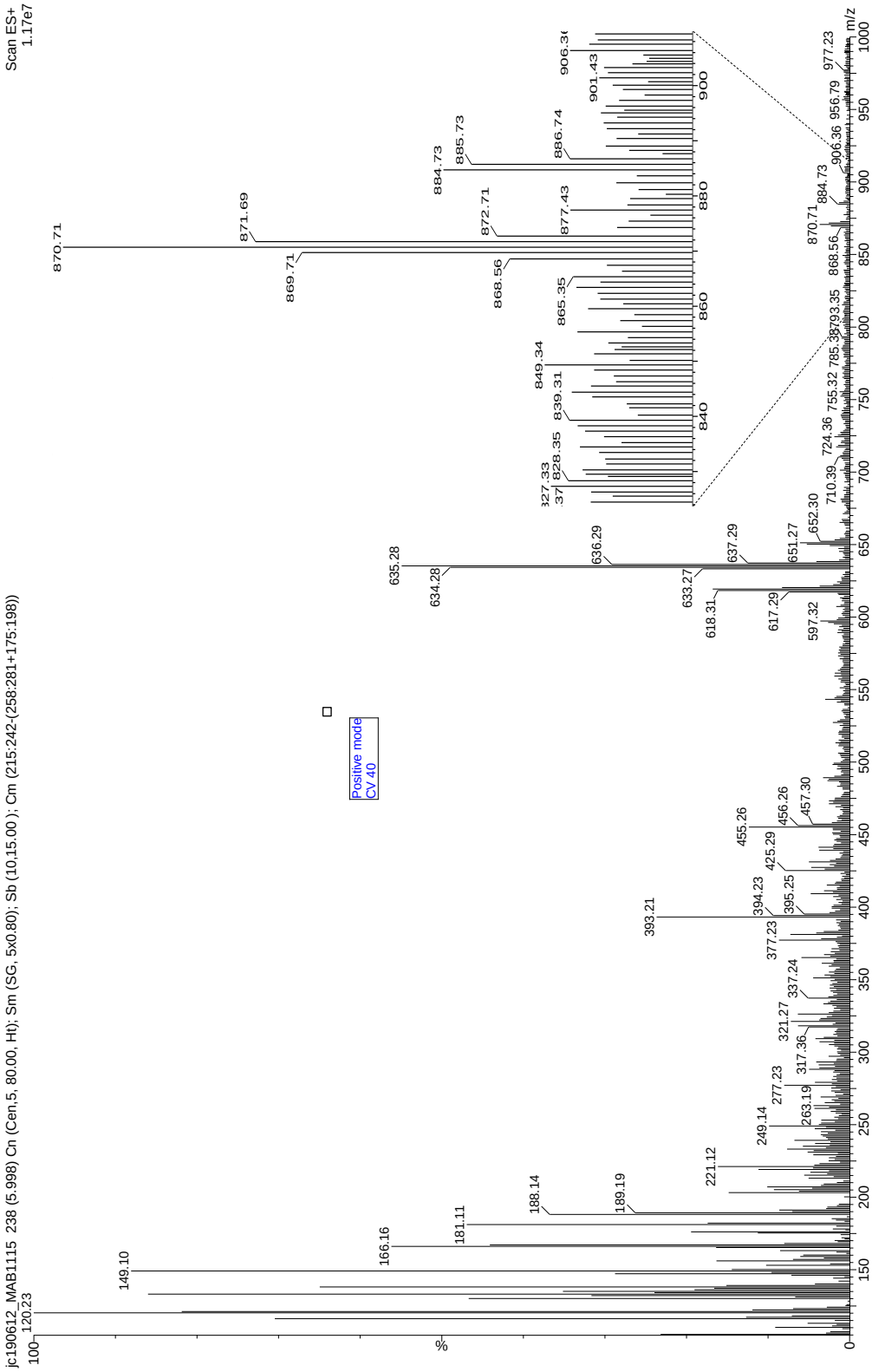


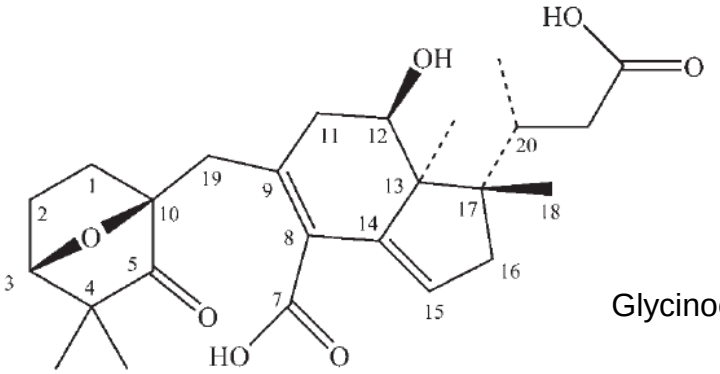
Figure 6.14. Positive ion ESI-MS analysis of the aqueous extract from intact aerial part of *Solanum sisymbriifolium*

Discussion

6.1. Hatching ability of root exudate extracts

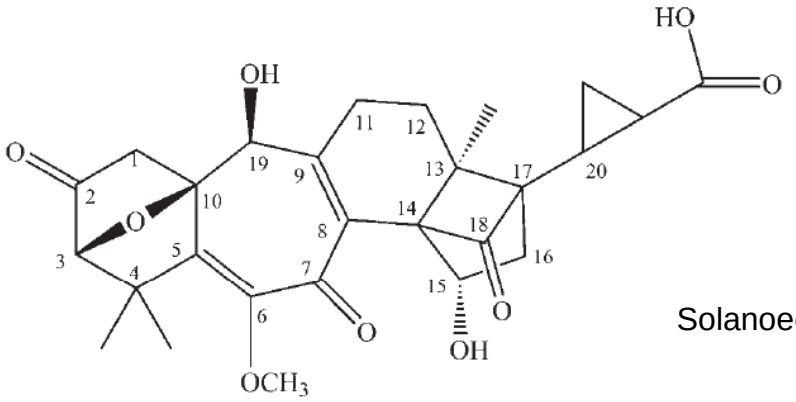
The first nematode hatching factor to be isolated was glycinoeclepin A (Figure 6.15a), which was extracted from powdered kidney bean roots, rather than root exudates; it induced hatch of soybean cyst nematode, *Heterodera glycines* Ichinohe (Masamune *et al.*, 1982; Fukuzawa *et al.*, 1985a). Subsequently, two further natural hatching stimuli for *H. glycines* were isolated, and designated as glycinoeclepins B and C (Fukuzawa *et al.*, 1985b). The structure of solanoeclepin A (Figure 6.15b) has some resemblance to those of glycinoeclepins, hence it is so named (Schenk *et al.*, 1999).

The ESI-MS analysis confirmed that solanoeclepin A was present in the root exudate extract from hydroponically grown *S. tuberosum* L. cv. Desiree (Figure 6.1). Mulder *et al*. (1996) claimed that this hatching factor could be isolated from tomato roots as well as potato roots. Interestingly, Devine and Jones (2000a) reported that the molecular weight of the hatching factors they isolated from potato plants cv. Kerr’s Pink was 530.5, which is higher than that of solanoeclepin A (498.5) (Mulder *et al.*, 1996). Devine and Jones (2000a) inferred that this difference might reflect the fact that the root exudate source to isolate HFs was different: potato for them, whereas tomato for Mulder *et al*. (1996). However, Schenk *et al*. (1999), who determined the structure of solanoeclepin A from potato, claimed the structure of the hatching factor isolated from tomato root exudates was identical to that from potato.



Glycinoeclepin A

(a)



Solanoeclepin A

(b)

Figure 6.15. Similarity between (a) glycinoeclepin A and (b) solanoeclepin A.

The confirmation of the presence of solanoeclepin A in the root exudate extract from *S. tuberosum* Desiree in section 6.1.1 proved that method employed (SPE of the liquid medium, methanol extraction, methanol removal, ESI-MS analysis with positive ionisation) was correct to detect solanoeclepin A. The same method was applied to *S. sisymbriifolium*, but the ESI-MS analysis suggested the absence of solanoeclepin A in the root exudate extract from hydroponically grown *S. sisymbriifolium* (Figure 6.2).

In order to find out if *S. sisymbriifolium* exudes a different HF from that of *S. tuberosum* Desiree, reversed-phase HPLC fractions (A, B, C and D, comprising 15 min aliquots) of the root exudate extracts from both *Solanum* species were obtained and subjected to hatching assays to investigate which fraction retained hatching ability comparable to the unfractionated root exudate extract. The results showed that fraction C with *S. tuberosum* L. cv. Desiree and fraction B with *S. sisymbriifolium* retained the ability, indicating a HF differs between the two *Solanum* species (Figure 6.4). This reinforced the finding in section 6.1.1 of the absence of solanoeclepin A in the root exudate extract from *S. sisymbriifolium,* and therefore the hypothesis that “a potato-derived HF, solanoeclepin A, is exuded from the roots of *S. sisymbriifolium*” was rejected.

With *S. tuberosum* Desiree*,* fraction A also showed strong hatching ability that was not significantly different from that of fraction C (Figure 6.4). It can be postulated that solanoeclepin A is present in fraction C, and that the potato glycoalkaloids (solanine and chaconine) that are natural HFs (Devine *et al.*, 1996; Byrne *et al.*, 1998) are in fraction A. Solanoeclepin A is more likely to be eluted in fraction C rather than A, due to its more lipophilic structure than the glycoalkaloids. Interestingly, in ESI-MS analysis of the root exudate of *S. sisymbriifolium*, neither solanine ($C_{45}H_{73}NO_{15}$, molecular weight = 868.06) nor chaconine ($C_{45}H_{73}NO_{14}$, molecular weight = 852.06) was detected (Figure 6.16), which may explain why fraction A of *S. sisymbriifolium* did not induce strong hatching at all unlike *S. tuberosum* Desiree (Figure 6.4). Therefore, with *S. sisymbriifolium* it can be suggested that fraction B contains a HF.

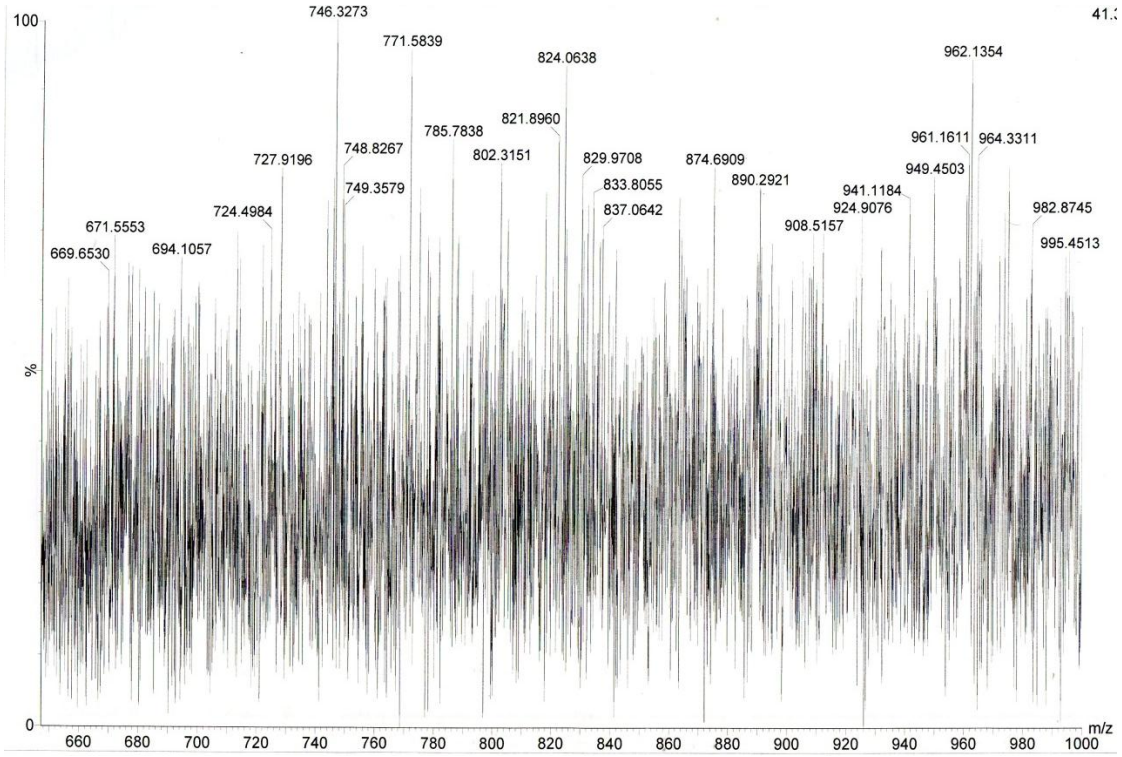


Figure 6.16. Positive ion ESI-MS analysis of *Solanum sisymbriifolium* root exudate extract. There is no peak to suggest the presence of solanine (molecular weight = 868.06) or chaconine (molecular weight = 852.06).

In order to investigate a HF in fraction B of *S. sisymbriifolium* root exudate extract, a series of bioassay-guided sub-fractionations were conducted (Figures 6.5 and 6.7). The results narrowed down a fraction that contained a HF to B1-d (comprising 1 min aliquot).

Effort was made to identify a HF in fraction B1-d by using ^{1}H-NMR and ESI-MS analyses, but due to an insufficient quantity of sample, there were no detectable signals to report.

6.2. Hatching ability of aerial part extract

6.2.1. Ethanol extract from aerial part of *S. sisymbriifolium*

6.2.2. Aqueous extract from macerated aerial part of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

The ethanol extract of the aerial part of *S. sisymbriifolium*, provided by Branston Ltd., showed significant hatching ability at 5-fold-water dilution (Figure 6.8). This result prompted further investigation of the hatching ability of the aqueous extract of the aerial part. Since a field practice for *S. sisymbriifolium* is to chop it up and plough it into the soil as green manure after growth, it was decided to obtain aqueous extract from the macerated aerial part of both *S. sisymbriifolium* and *S. tuberosum* Desiree, in order to reproduce the field situation.

The result of hatching assays showed that *S. tuberosum* Desiree had a fairly good hatching ability at every dose without significant difference between doses, although not comparable to that of PRD at any dose (Figure 6.9). With *S. sisymbriifolium* the undiluted extract hardly induced hatching, but with dilution hatching ability started to manifest and peaked at 10 mg/ml, where hatching ability was as strong as that of PRD (Figure 6.9). The result indicates that the “chopped up and ploughed in” practice, which has been applied to *S. sisymbriifolium* after growth in the field, may have been unwittingly contributing to further reduction of PCN population in the field by inducing additional hatching. It can be suggested that this practice of “chopped up and ploughed in as green manure” should be extended to potato as well.

This is the first study that has reported the hatching ability of macerated aerial parts of either *S. tuberosum* or *S. sisymbriifolium.*

6.2.3. Aqueous extract from intact aerial part of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

Unexpectedly the aqueous extract from intact aerial part of *S. sisymbriifolium* proved to stimulate very strong hatching of *G. pallida* J2s (Figure 6.10).

Since under field conditions rain water runs through the aerial part of potato crop and soaks into the soil, Devine *et al*. (1996) investigated how water-soluble materials leached from the aerial part of potato would contribute to hatching of *G. rostochiensis*. The authors found out that the hatching activity from the combined leachates (root and aerial part) was similar to that from the root leachate on its own, except at the highest concentration, where the activity was significantly greater than that of the root leachate, even though the aerial part leachate showed low hatching activity at every concentration. Therefore, the authors inferred that there is synergism between hatching chemicals from the two leachates.

The current study examined the hatching activity from the aerial part of *S. sisymbriifolium* and *S. tuberosum* L. cv. Desiree in a slightly different way from Devine *et al*. (1996): the aerial part was immersed in distilled water for 48 h, instead of 1 h with these authors, and the aqueous extract was subjected to freeze-drying, instead of rotary evaporation. Even with this longer period of time

for immersing the aerial part in this study, the hatching ability of *S. sisymbriifolium* as high as that of PRD at three doses (Figure 6.10) was not expected, because of the result shown by Devine *et al.* (1996) with potato.

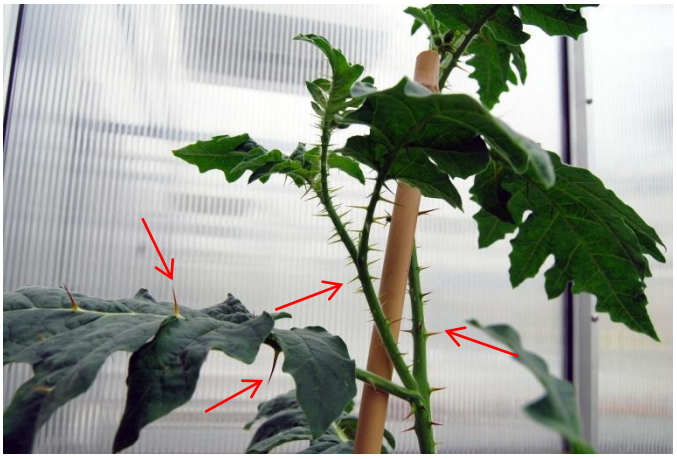
Interestingly although the fresh weight of the aerial part used for soaking was heavier with *S. tuberosum* Desiree (51.7 g and 43.1 g for *S. tuberosum* Desiree and *S. sisymbriifolium*, respectively), the dry matter yielded from freeze-drying of the aqueous extract was less with *S. tuberosum* Desiree (0.535 g and 0.6 g for *S. tuberosum* Desiree and *S. sisymbriifolium*, respectively). This suggests the aerial part of *S. sisymbriifolium* has more water-soluble compounds per gram of fresh weight than *S. tuberosum* Desiree. This was confirmed in the reversed-phase HPLC profiles shown in Figure 6.11, where much higher activities are demonstrated with *S. sisymbriifolium* especially at early retention times. This may be reflected in the significantly stronger hatching ability of *S. sisymbriifolium* at the three highest doses than *S. tuberosum* Desiree (Figure 6.10). At these doses, hatching ability of *S. sisymbriifolium* was statistically as high as that of PRD (Figure 6.10), even though its aerial part was simply immersed in water. Unlike the macerated aerial part extract in section 6.2.2, hatching ability of aqueous extract from the intact aerial part of *S. tuberosum* Desiree showed a dosage-responsive curve (Figure 6.10), which peaked at 16 mg/ml (fresh weight) and gradually declined: the hatching ability at 16 mg/ml was significantly higher than that at 80 mg/ml and also than that at 0.8 mg/ml, although still significantly lower than that of PRD.

The result demonstrates a clear difference in hatching ability between *S. sisymbriifolium* and *S. tuberosum* Desiree. There are two interesting

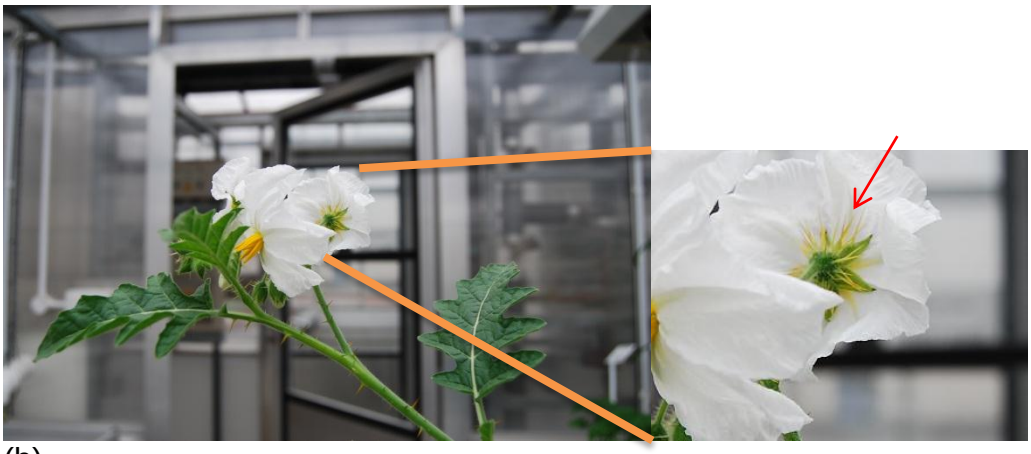
characteristics regarding the aerial part of *S. sisymbriifolium,* both of which are missing in *S. tuberosum:* i) it is covered with thorns: leaves (both the adaxial and abaxial sides), stems and even calyxes are covered with thorns as shown in Figure 6.17; ii) it is sticky wherever one touches: its common name "sticky nightshade" must derive from this aspect. Presumably both features are part of defence strategies: thorns against large herbivores; stickiness against small insects. For another Solanaceous species, *S. berthaultii* Hawkes (the wild potato plant), Gibson and Turner (1977) reported that its leaves had two types of glandular trichomes: a short type with a four-lobed gland at its apex (type A), and a longer multicellular trichome with an ovoid gland at its tip (type B). It is reported that type A trichomes, when ruptured, release the enzyme polyphenoloxidase which rapidly polymerises the substrate in the trichome head (Ryan *et al.*, 1982), whereas type B trichomes with an ovoid gland continually discharge a clear viscous exudate whose main components are fatty acid esters of sucrose (Gibson & Turner, 1977; King *et al.*, 1986; 1987a; 1987b). It has been shown that as well as a non-volatile constituent, the exudate from type B trichomes of *S. berthaultii* also contains many volatile sesquiterpenes, *e.g.* the aphid alarm pheromone (E)-β-farnesene (Gibson & Pickett, 1983).

Hill *et al*. (1997), who examined the leaves of several *Solanum* species (3 South African indigenous and 4 South American species), showed that some of those plant species tested, including *S. sisymbriifolium,* had only type B glandular trichomes (Figure 6.18), and with *S. sisymbriifolium* the densities of type B trichomes were much higher than other *Solanum* species tested. This is in agreement with the results from the ^{1}H-NMR (Figure 6.12) and ESI-MS

analyses (Figure 6.13), which suggest the strong presence of sugar units. As shown with other *Solanum* species (King *et al.*, 1986; King *et al.*, 1987a; King *et al.*, 1987b), it is likely that the major components of the exudates from type B trichomes of *S. sisymbriifolium* are also acyl-sugars (sucrose esters). Interestingly King *et al*. (1986) inferred that the high resistance exhibited by clone 1726 of *S. berthaultii* against *Phytophthora infestans* might be attributed to the high densities of type B trichomes on its leaves that constantly exude sucrose esters. *Solanum sisymbriifolium* is reported to be highly resistant to *P. infestans* (Timmermans, 2005), whereas *Solanum tuberosum,* which lacks both type A and type B glandular trichomes (Neal *et al.*, 1989), is susceptible. ^{1}H-NMR (Figure 6.12) and the subsequent ESI-MS analyses (Figure 6.13) of *S. sisymbriifolium* aqueous aerial extract (intact) in this current study indicated the presence of chlorogenic acid. Interestingly it has been reported that the press juice of potato leaves of a resistant cultivar (Aquila) totally inhibited *P. infestans,* and it was due to a high concentration of chlorogenic acid (Virtanen *et al.*, 1957; Valle, 1957). Chlorogenic acid has also been shown to play an important role in reducing infection with fungal pathogens, such as *Monilinia laxa* (Aderh. and Ruhl.) (brown rot) and *Cercospora nicotianae* (Ell. and Ev.) in peach fruits and tobacco leaves, respectively (Shadle *et al.*, 2003; Villarino *et al.*, 2011). It can be speculated that high density of type B trichomes coupled with a high level of chlorogenic acid in the leaves of *S. sisymbriifolium* contribute to the high resistance against *P. infestans.*

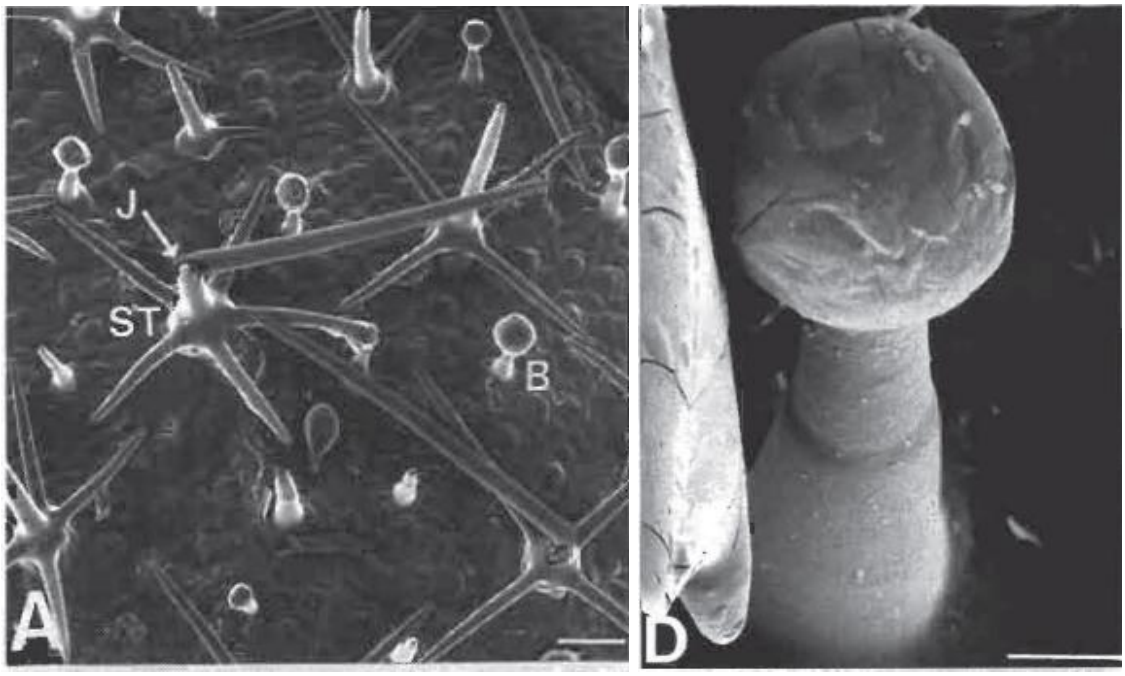


(a)



(b)

Figure 6.17. Thorns of *Solanum sisymbriifolium.* (a) On leaves and stems (*arrow*), (b) on the calyx (*arrow*).



(a) (b)

Figure 6.18. “Scanning electron micrograph of: (a) the leaf surface of *Solanum sisymbriifolium* with the central ray of the glandular stellate trichome broken at the joint. ST: stellate trichome, B: type B glandular trichome, J: joint in trichome. Scale bar = 100 µm; (b) a type B glandular trichome on the leaf of *S. sisymbriifolium.* Scale bar = 10 µm”. Both (a) and (b) were reproduced with permission of publishers from: Hill, M.P., Hulley, P.E., Allsopp, J. & Van Harmelen, G. 1997. Glandular trichomes on the exotic *Solanum sisymbriifolium* Lamarck (Solanaceae): Effective deterrents against an indigenous South African herbivore. *African Entomology* **5**(1): 41–50.

It is possible that one of the compounds that are designed to defend the plant from pathogen or insect attacks happens to induce hatching of *G. pallida* J2s. In order to identify a HF of *S. sisymbriifolium* (aerial part), bioassay-guided fractionations need to be undertaken.

This study is the first to reveal notable ability of the aerial part of *S. sisymbriifolium* to induce strong hatching of *G. pallida*, which was demonstrated in three different extracts: ethanol extract, aqueous extract from macerated and intact aerial part. Identification of the HF of *S. sisymbriifolium* will provide

underpinning science for the application of aerial part of *S. sisymbriifolium* in novel trap crop control of PCN.

Chapter 7

General Discussion

The most recent survey by Minnis *et al.* (2002) revealed that potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, were present in 64 % of the sites sampled in England and Wales, which was a significant increase from 42 % from the previous survey by Hancock (1996). Minnis *et al.* (2002) found *G. pallida* and *G. rostochiensis* in 92 % and 33 % of samples, respectively, which was contrary to earlier surveys in the 1960s that revealed approximately half of the PCN populations in England were pure *G. rostochiensis* (Brown, 1970). The suggestion is that repeated cropping with cultivars that are resistant to *G. rostochiensis* but not to *G. pallida*, such as Maris Piper, has selected *G. pallida* (Minnis *et al.*, 2002).

Unfortunately, as explained in Chapter 1. General Introduction, it is more difficult to control *G. pallida*, because of the following reasons: i) cultivars with only partial resistance are available, with no major gene resistance to *G. pallida* identified (Phillips & Blok, 2008); ii) *G. pallida* hatch more slowly than *G. rostochiensis* (Whitehead, 1992), which makes control by nematicides more difficult; iii) the rate of spontaneous hatching of *G. pallida* in absence of the host is much lower than *G. rostochiensis* (Greet, 1974; Den Nijs & Lock, 1992), which makes control by rotations more difficult.

Integrated control measures combining the use of partially resistant cultivars, longer rotations and effective nematicides have to be applied to control *G. pallida*. However, effective nematicides either have been, or are about to be, withdrawn, due to the Montreal Protocol and the European Council Directive 91/414/EEC on environmental and health grounds as explained in section 1.4.2 of Chapter 1.

Under these circumstances, the trap crop *Solanum sisymbriifolium*, which induces strong hatch of, and is totally resistant to, both *G. rostochiensis* and *G. pallida,* has been used by some growers in the UK and The Netherlands to reduce the field population. Although effective and attractive as an environmentally benign strategy, growing *S. sisymbriifolium* requires the same attention and practice as those for crops. With its medicinal value, it is feasible to make growing *S. sisymbriifolium* an attractive commercial option for growers. However, if the mode of action of this unique trap crop can be discovered, it could lead to a novel control strategy for PCN.

Bioassays conducted to investigate the mode of action of *S. sisymbriifolium* revealed interesting insights of the behaviour of *G. pallida* J2s towards plants. First of all, as shown in choice (attraction) assays (section 3.1.2 of Chapter 3), unexpectedly *G. pallida* J2s were equally attracted (taxis*) to the host (*S. tuberosum* Desiree) and to the trap crop (*S. sisymbriifolium*), and were even more attracted to the known non-host (wheat) than to the host in terms of the number of the nematodes which moved toward the non-host. The difference was, however, the speed of nematode movement towards the plantlets, which can be described as the host > the trap crop > the non-host. This is the first study to reveal that *G. pallida* J2s are attracted to a non-host plant (wheat) as well as to the trap crop *S. sisymbriifolium.*

Interestingly, although a living plant of *S. tuberosum* Desiree attracted *G. pallida* J2s, its root diffusate (PRD) was not attractive, as shown in chemotaxis assays (section 3.2 of Chapter 3). However, the J2s that hatched in PRD

* See Glossary for definition.

showed faster orthokinesis* (relating to speed of non-directional movement) than those that hatched in water (see section 3.3 of Chapter 3). These results, coupled with those from choice (attraction) assays (section 3.1.2), suggest that a *S. tuberosum* Desiree plant induces both orthokinesis (speed of non-directional movement) and taxis (directed movement, attraction).

For invasion, also unexpectedly, significantly more *G. pallida* J2s invaded per gram root of *S. sisymbriifolium* than *S. tuberosum* L. cv. Desiree at every time point (1, 2, 3 and 4 dpi) as shown in section 3.4.1 of Chapter 3. By contrast, *G. pallida* J2s did not invade the roots of the known non-host wheat, even though being strongly attracted to its roots, which suggests that a short-distant attractant* for *G. pallida* is generic but a local attractant* is species-specific.

However, for development, *G. pallida* J2s that invaded the roots of *S. sisymbriifolium* failed to develop further. By 14 dpi, the difference in nematode development between the trap crop and the host became evident as shown in section 3.4.2 of Chapter 3. The unique characteristics of *S. sisymbriifolium* seem to be that the immunity to *G. pallida* infection appears to be 100 %, because no adult females or adult males develop. This is totally unlike other incompatible* interactions, because some cysts always develop on any known resistant cultivars (even though significantly fewer than on a susceptible plant) and also it is common to find many adult males on resistant species, as described in section 3.4 of Discussion of Chapter 3.

* See Glossary for definition.

This study demonstrated for the first time that Pluronic F-127 aqueous solution is an ideal medium for *in vitro* development bioassays of *G. pallida* J2s, which can facilitate continuous observation of nematode development under non-sterile conditions (see Chapter 4).

The possibility that invaded J2s subsequently emerged from the roots of *S. sisymbriifolium* was rejected on two accounts: i) there was no sign of obvious decline in the number of the nematodes within the roots in the development assay as shown in section 3.4.2 of Chapter 3; ii) the pilot experiment to assess re-emergence of the nematodes, for which the method described in Chapter 4 was employed, revealed no sign of it as shown in section 5.1 of Chapter 5. It is an interesting contrast to the incompatible interaction between another PCN species, *G. rostochiensis,* and a potato cultivar (Maris Piper) with the *H1* gene that confers resistance to *G. rostochiensis,* where Forrest *et al.* (1986) revealed that invaded *G. rostochiensis* J2s subsequently emerged from the roots of Maris Piper.

If *G. pallida* J2s are attracted to the roots of *S. sisymbriifolium*, invade them in large numbers and yet do not leave the roots, another possibility could be that the plant produces toxic compounds to paralyse or kill the invaded nematodes. This possibility was also rejected in section 5.1 of Chapter 5, as it was observed that invaded nematodes that were subsequently exposed from the roots were moving.

In order to investigate the possibility that the immunity of *S. sisymbriifolium* is due to up-regulation of defence-related genes, the changes in the gene expression within the roots were assessed during infection with *G.*

pallida J2s by quantitative real-time polymerase chain reaction (qRT-PRC) (see section 5.2 of Chapter 5). The results revealed that the chitinase gene (*ChtC 2.1*, a potato class I basic chitinase) was significantly up-regulated at 3 dpi in the roots of *S. sisymbriifolium* but not in *S. tuberosum* Desiree. At 10 dpi, although up-regulation of the chitinase gene (*ChtC 2.1*) was observed in infected *S. tuberosum* Desiree, the magnitude of gene expression increase from the uninfected was only one third of that observed for *S. sisymbriifolium*. The up-regulation of the chitinase gene (*Chi9*, a tomato class I basic chitinase) has been shown in a resistant tomato cultivar at 3 dpi with *G. rostochiensis,* but the expression subsequently decreased to the level of uninfected at 7 dpi (Uehara *et al.*, 2010). On this resistant tomato, the authors found some cysts developed, though significantly fewer than on a susceptible tomato. By contrast, no cysts developed on *S. sisymbriifolium* in this study and also in studies by Roberts & Stone (1983) and Scholte (2000c). It can be speculated that the strong expression of the chitinase gene (*ChtC 2.1*) not only at 3 dpi but also at 10 dpi with an even higher level than 3 dpi may contribute to the total immunity of *S. sisymbriifolium* to *G. pallida.* This finding might lead to development of a transgenic potato with total immunity to PCN.

In this study, it was hypothesised that solanoeclepin A, a HF of *S. tuberosum,* is also exuded by the roots of *S. sisymbriifolium,* but this hypothesis was rejected following these findings: i) ESI-MS analysis suggested the absence of solanoeclepin A in the root exudate extract from *S. sisymbriifolium* (see section 6.1.1 of Chapter 6); ii) subsequent hatching assays with HPLC fractions of the root exudate extracts revealed that a HF is present in different

fractions between the two *Solanum* species (see section 6.1.2.2 of Chapter 6), indicating a HF differs between them. Due to the lack of sufficient quantity of the sample material, identification of a HF of *S. sisymbriifolium* did not materialise. However, unexpectedly an aqueous extract from the intact aerial part of *S. sisymbriifolium* proved to have a significant hatching ability, comparable to that of potato root diffusate (PRD) (see section 6.2.3 of Chapter 6). Also with macerated aerial part (aqueous) extract, a certain dilution showed a strong hatching activity comparable to that of PRD (see section 6.2.2 of Chapter 6). These results revealed a potential unexpected effect of using the aerial part of *S. sisymbriifolium* as green manure after growth on further reduction of PCN populations in the field. This may provide a strong incentive to growers to use *S. sisymbriifolium* as a trap crop for PCN, but identification of the HF of *S. sisymbriifolium* could make an even more attractive option possible, that is inducing suicide hatch without the need to plant *S. sisymbriifolium.*

Conclusion

This project proved that *S. sisymbriifolium* is an excellent trap crop, as it attracts *G. pallida* J2s strongly, allows liberal invasion and yet totally inhibits their development at an early stage. Because of this early prevention of nematode development, neither adult females nor males of *G. pallida* develop in this plant, which is a unique quality of this plant and unlike any other incompatible interactions.

This study for the first time investigated expression changes of defence-related genes in *S. sisymbriifolium* roots in the time course infection with *G.*

pallida and showed a possibility that the chitinase gene (*ChtC 2.1*) plays an important part in the unique immunity of *S. sisymbriifolium* against *G. pallida.* This finding may contribute to a novel genetic intervention for controlling PCN.

Also revealed for the first time in this study was that the HF of *S. sisymbriifolium* is different from that of *S. tuberosum* (solanoeclepin A), and that aerial part of *S. sisymbriifolium* has a significantly strong hatching ability.

For future work, identification of the HF of *S. sisymbriifolium* will be beneficial for potato growers not only in the UK but also in other countries where other nematode species, such as *Meloidogyne hapla,* coexist with PCN, as planting *S. sisymbriifolium* in these countries might inadvertently increase other nematodes populations. Since the collection of sufficient quantities of extract from the aerial part of *S. sisymbriifolium* is much easier than collecting root exudate, there is a realistic hope to be able to identify the HF of *S. sisymbriifolium*.

Appendix I: Statistical analysis

3.1. Movement of *G. pallida* towards the roots

3.1.1. Separate assays for individual plant species (arrestment/attraction assays)

3.1.1.1. Comparison between *S. tuberosum* L. cv. Desiree (potato, host) and *S. sisymbriifolium* (trap crop)

Analysis of variance

Variate: LOG(Prop)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	4	1.3446	0.3361	1.78	
Block.PlantPair stratum					
Species	1	0.6784	0.6784	3.60	0.131
Residual	4	0.7542	0.1885	1.81	
Block.PlantPair.Time stratum					
Time	3	44.8036	14.9345	143.61	<.001
Time.Species	3	0.3769	0.1256	1.21	0.328
Residual	24	2.4959	0.1040		
Total	39	50.4536			

Tables of means

Variate: LOG(Prop)

Grand mean 1.687

Time	0	30	60	120
	-0.142	2.178	2.325	2.386

Species	Ss	St
	1.557	1.817

Time	Species	Ss	St
0		-0.335	0.051
30		1.968	2.388
60		2.359	2.291
120		2.234	2.537

Standard errors of differences of means

Table	Time	Species	Time Species
rep.	10	20	5
s.e.d.	0.1442	0.1373	0.2237
d.f.	24	4	19.36
Except when comparing means with the same level(s) of Species			0.2040
d.f.			24

Least significant differences of means (5% level)

Table	Time	Species	Time Species
rep.	10	20	5
l.s.d.	0.2977	0.3812	0.4677
d.f.	24	4	19.36
Except when comparing means with the same level(s) of Species			0.4209
d.f.			24

3.1.1.2. Comparison between *S. sisymbriifolium* (trap crop) and wheat (*Triticum Aestivum*, non-host)

Analysis of variance

Variate: LOG(Ratio)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	3	0.60189	0.20063	0.43	
Block.Plant_pair stratum					
Plant_species	1	0.11387	0.11387	0.24	0.655
Residual	3	1.40193	0.46731	11.87	
Block.Plant_pair.Time stratum					
Time	3	14.84099	4.94700	125.62	<.001
Time.Plant_species	3	0.07277	0.02426	0.62	0.614
Residual	18	0.70887	0.03938		
Total	31	17.74033			

Tables of means

Variate: LOG(Ratio)

Grand mean 1.102

Time	0	30	60	120
	-0.074	1.400	1.526	1.555

Plant_species	Ss	Wheat
	1.161	1.042

Time	Plant_species	Ss	Wheat
0		-0.081	-0.066
30		1.481	1.319
60		1.648	1.404
120		1.598	1.511

Standard errors of differences of means

Table	Time	Plant_species	Time Plant_species
rep.	8	16	4
s.e.d.	0.0992	0.2417	0.2705
d.f.	18	3	4.66
Except when comparing means with the same level(s) of Plant_species			0.1403
d.f.			18

Least significant differences of means (5% level)

Table	Time	Plant_species	Time Plant_species
rep.	8	16	4
l.s.d.	0.2085	0.7692	0.7110
d.f.	18	3	4.66
Except when comparing means with the same level(s) of Plant_species			0.2948
d.f.			18

3.1.2. Choice assays between two plant species (attraction assays)

3.1.2.1. Between *S. tuberosum* L. cv. Desiree (host) and *S. sisymbriifolium* (trap crop)

Estimates of parameters

Parameter	estimate	s.e.	t(88)	t pr.	antilog of estimate
Constant	2.443	0.555	4.40	<.001	11.51
Plant_species St	0.023	0.779	0.03	0.976	1.024
Time_min	-0.00028	0.00604	-0.05	0.963	0.9997
Distance.Plant_species Ss	-2.203	0.590	-3.74	<.001	0.1105
Distance.Plant_species St	-1.752	0.505	-3.47	<.001	0.1735
Time_min.Plant_species St	-0.00966	0.00885	-1.09	0.278	0.9904
Time_min.Distance.Plant_species Ss	0.01204	0.00608	1.98	0.051	1.012
Time_min.Distance.Plant_species St	0.01454	0.00553	2.63	0.010	1.015

Accumulated analysis of deviance

Change	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
+ Plant_species	1	0.915	0.915	0.74	0.393
+ Time_min	1	20.328	20.328	16.36	<.001
+ Distance.Plant_species	2	63.750	31.875	25.65	<.001
+ Time_min.Plant_species	1	1.913	1.913	1.54	0.218
+ Time_min.Distance.Plant_species	2	13.821	6.910	5.56	0.005
Residual	88	109.344	1.243		
Total	95	210.070	2.211		

Predictions from regression model

	Plant_species	Ss		St	
		Prediction	s.e.	Prediction	s.e.
Time_min	Distance				
30	0.5	4.543	1.0372	4.528	1.0658
	1.0	1.809	0.3615	2.345	0.4197
	1.5	0.720	0.2455	1.215	0.3254
	2.0	0.287	0.1520	0.629	0.2629
60	0.5	5.396	0.7936	4.179	0.6463
	1.0	2.574	0.3301	2.692	0.3131
	1.5	1.228	0.2682	1.734	0.2977
	2.0	0.586	0.1990	1.117	0.2992
90	0.5	6.409	0.7929	3.856	0.6028
	1.0	3.662	0.3462	3.089	0.3269
	1.5	2.093	0.2980	2.475	0.3036
	2.0	1.196	0.2656	1.983	0.3762
120	0.5	7.613	1.3860	3.559	0.8491
	1.0	5.211	0.6876	3.546	0.5611
	1.5	3.567	0.6566	3.533	0.5962
	2.0	2.442	0.7017	3.520	0.9127

3.1.2.2. Between *S. tuberosum* L. cv. Desiree (host) and wheat (non-host)

Estimates of parameters

Parameter	estimate	s.e.	t(88)	t pr.	antilog of estimate
Constant	3.366	0.321	10.49	<.001	28.97
Plant_species Wheat	0.507	0.420	1.21	0.231	1.661
Time	-0.00396	0.00366	-1.08	0.281	0.9960
Distance.Plant_species St	-1.964	0.326	-6.03	<.001	0.1403
Distance.Plant_species Wheat	-1.898	0.273	-6.95	<.001	0.1499
Time.Plant_species Wheat	-0.00429	0.00490	-0.88	0.384	0.9957
Time.Distance.Plant_species St	0.01123	0.00350	3.21	0.002	1.011
Time.Distance.Plant_species Wheat	0.01089	0.00308	3.54	<.001	1.011

Accumulated analysis of deviance

Change	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
+ Plant_species	1	7.102	7.102	6.92	0.010
+ Time	1	13.030	13.030	12.70	<.001
+ Distance.Plant_species	2	208.815	104.407	101.78	<.001
+ Time.Plant_species	1	4.045	4.045	3.94	0.050
+ Time.Distance.Plant_species	2	23.690	11.845	11.55	<.001
Residual	88	90.271	1.026		
Total	95	346.952	3.652		

Predictions from regression model

Time	Plant_species Distance	St Prediction	s.e.	Wheat Prediction	s.e.
30	0.5	11.405	1.5246	17.121	1.9170
	1.0	5.057	0.5609	7.806	0.7190
	1.5	2.242	0.4084	3.559	0.5348
	2.0	0.994	0.2821	1.622	0.3799
60	0.5	11.985	1.0403	15.737	1.1615
	1.0	6.289	0.4494	8.448	0.5058
	1.5	3.300	0.3851	4.535	0.4376
	2.0	1.732	0.3149	2.434	0.3662
90	0.5	12.594	1.0139	14.464	1.1082
	1.0	7.822	0.4604	9.143	0.5050
	1.5	4.858	0.4041	5.779	0.4404
	2.0	3.017	0.3916	3.653	0.4343
120	0.5	13.234	1.6051	13.295	1.5620
	1.0	9.728	0.8356	9.895	0.8211
	1.5	7.151	0.8182	7.364	0.8090
	2.0	5.257	0.9382	5.481	0.9390

3.1.2.3. Between *S. sisymbriifolium* (trap crop) and wheat (non-host)

Estimates of parameters

Parameter	estimate	s.e.	t(88)	t pr.	antilog of estimate
Constant	2.925	0.599	4.88	<.001	18.63
Plant_species Wheat	-0.235	0.833	-0.28	0.778	0.7904
Time	-0.00055	0.00647	-0.09	0.932	0.9994
Distance.Plant_species Ss	-2.527	0.678	-3.73	<.001	0.07991
Distance.Plant_species Wheat	-2.054	0.605	-3.40	0.001	0.1282
Time.Plant_species Wheat	0.00012	0.00909	0.01	0.989	1.000
Time.Distance.Plant_species Ss	0.01306	0.00693	1.89	0.063	1.013
Time.Distance.Plant_species Wheat	0.01062	0.00635	1.67	0.098	1.011

Accumulated analysis of deviance

Change	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
+ Plant_species	1	0.003	0.003	0.00	0.970
+ Time	1	40.180	40.180	22.00	<.001
+ Distance.Plant_species	2	157.642	78.821	43.16	<.001
+ Time.Plant_species	1	0.177	0.177	0.10	0.756
+ Time.Distance.Plant_species	2	12.063	6.032	3.30	0.041
Residual	88	160.712	1.826		
Total	95	370.777	3.903		

Predictions from regression model

Time	Distance	Plant_species Ss Prediction	s.e.	Wheat Prediction	s.e.
30	0.5	6.301	1.4945	6.104	1.4623
	1.0	2.167	0.4912	2.563	0.5258
	1.5	0.745	0.3018	1.077	0.3715
	2.0	0.256	0.1609	0.452	0.2428
60	0.5	7.538	1.1516	7.066	1.0919
	1.0	3.154	0.4587	3.480	0.4583
	1.5	1.319	0.3426	1.714	0.3787
	2.0	0.552	0.2223	0.844	0.2903
90	0.5	9.019	1.1545	8.179	1.0885
	1.0	4.590	0.4762	4.723	0.4761
	1.5	2.336	0.3888	2.728	0.4100
	2.0	1.189	0.3081	1.575	0.3695
120	0.5	10.791	2.0304	9.468	1.8669
	1.0	6.680	0.9499	6.412	0.9185
	1.5	4.136	0.8760	4.342	0.8722
	2.0	2.560	0.8466	2.941	0.9223

3.1.2.4. Between *S. tuberosum* L. cv. Desiree (host) and “no plant”

Estimates of parameters

Parameter	estimate	s.e.	t(88)	t pr.	antilog of estimate
Constant	1.959	0.917	2.14	0.035	7.092
Plant_species St	1.40	1.05	1.33	0.186	4.057
Time	-0.0141	0.0102	-1.38	0.171	0.9860
Distance.Plant_species no plant	-2.765	0.924	-2.99	0.004	0.06295
Distance.Plant_species St	-3.068	0.598	-5.13	<.001	0.04653
Time.Plant_species St	0.0055	0.0116	0.47	0.640	1.005
Time.Distance.Plant_species no plant	0.02606	0.00919	2.83	0.006	1.026
Time.Distance.Plant_species St	0.02095	0.00598	3.51	<.001	1.021

Accumulated analysis of deviance

Change	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
+ Plant_species	1	58.277	58.277	46.49	<.001
+ Time	1	27.744	27.744	22.13	<.001
+ Distance.Plant_species	2	66.422	33.211	26.49	<.001
+ Time.Plant_species	1	0.633	0.633	0.51	0.479
+ Time.Distance.Plant_species	2	27.416	13.708	10.93	<.001
Residual	88	110.320	1.254		
Total	95	290.812	3.061		

Predictions from regression model

	Plant_species	No plant		St	
		Prediction	s.e.	Prediction	s.e.
Time	Distance				
30	0.5	1.725	0.6671	6.565	1.3078
	1.0	0.640	0.2084	1.939	0.3898
	1.5	0.237	0.1266	0.573	0.2093
	2.0	0.088	0.0728	0.169	0.0956
60	0.5	1.672	0.4222	6.942	0.8977
	1.0	0.917	0.1930	2.808	0.3634
	1.5	0.503	0.1730	1.136	0.2670
	2.0	0.276	0.1474	0.459	0.1671
90	0.5	1.621	0.3819	7.342	0.8610
	1.0	1.314	0.2146	4.066	0.3735
	1.5	1.065	0.2104	2.252	0.3171
	2.0	0.863	0.2625	1.247	0.2730
120	0.5	1.571	0.5552	7.765	1.3622
	1.0	1.883	0.4341	5.888	0.7328
	1.5	2.256	0.4854	4.465	0.7371
	2.0	2.704	0.8730	3.386	0.8703

3.2. Chemotaxis of *G. pallida* towards root exudates of *S. tuberosum* L. cv. Desiree

Assays with 1% AcOH, PRD (undiluted) and distilled water (DW)

Analysis of variance

Variate: Cf

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treat	2	1.25347	0.62674	45.12	<.001
Residual	6	0.08333	0.01389		
Total	8	1.33681			

Tables of means

Variate: Cf

Grand mean 0.597

Treat	1% AcOH	DW	PRD (neat)
	0.083	0.958	0.750

Standard errors of differences of means

Table	Treat
rep.	3
d.f.	6
s.e.d.	0.0962

Least significant differences of means (5% level)

Table	Treat
rep.	3
d.f.	6
l.s.d.	0.2355

Assays with 0.5 M $CaCl_2$, two-fold concentrated PRD, two-fold diluted PRD (1:1), four-fold diluted PRD (1:3) and tap water (TW)

Analysis of variance

Variate: Cf

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	4	0.41436	0.10359	1.65	0.237
Residual	10	0.62717	0.06272		
Total	14	1.04154			

Tables of means

Variate: Cf

Grand mean 1.003

Treatments	0.5 M CaCl2	PRD (1:1)
	1.314	0.828
Treatments	PRD (1:3)	TW
	1.006	0.911
Treatments	Two-fold concentrated PRD	
	0.958	

Standard errors of differences of means

Table	Treatments
rep.	3
d.f.	10
s.e.d.	0.2045

Least significant differences of means (5% level)

Table	Treatments
rep.	3
d.f.	10
l.s.d.	0.4556

3.3. Motility assays with root exudates of *S. tuberosum* L. cv. Desiree

3.3.1. Experiment with PRD-hatched *G. pallida* J2s

Analysis of variance

Variate: log_%_of_J2_100_%_of_J2 = log(%_of_J2/(100-%_of_J2))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Dish stratum					
Treatment	1	0.0099	0.0099	0.07	0.803
Residual	4	0.5531	0.1383	0.34	
Dish.Area stratum					
Area	3	98.7695	32.9232	81.57	<.001

Area.Treatment	3	0.3640	0.1213	0.30	0.824
Residual	12	4.8437	0.4036	3.66	
Dish.Area.Time_min stratum					
Time_min	2	0.1089	0.0544	0.49	0.615
Area.Time_min	6	17.4541	2.9090	26.38	<.001
Time_min.Treatment	2	0.2920	0.1460	1.32	0.280
Area.Time_min.Treatment	6	1.4296	0.2383	2.16	0.073
Residual	32	3.5290	0.1103		
Total	71	127.3536			

Tables of means

Variate: log_%_of_J2_100_%_of_J2

Grand mean -1.501

Area	0.0	0.5	1.5	2.5
	-3.355	-1.404	-0.123	-1.124

Time_min	30	60	90
	-1.467	-1.482	-1.556

Treatment	PRD (1:3)	Water
	-1.513	-1.490

Area	Time_min	30	60	90
0.0		-3.005	-3.444	-3.615
0.5		-0.723	-1.517	-1.973
1.5		0.026	-0.034	-0.361
2.5		-2.165	-0.932	-0.274

Area	Treatment	PRD (1:3)	Water
0.0		-3.485	-3.225
0.5		-1.407	-1.402
1.5		-0.087	-0.159
2.5		-1.074	-1.174

Time_min	Treatment	PRD (1:3)	Water
30		-1.395	-1.539
60		-1.506	-1.458
90		-1.639	-1.473

	Time_min	30		60		90	
Area	Treatment	PRD (1:3)	Water	PRD (1:3)	Water	PRD (1:3)	Water
0.0		-2.873	-3.138	-3.671	-3.217	-3.911	-3.318
0.5		-0.808	-0.638	-1.287	-1.746	-2.126	-1.820
1.5		-0.010	0.061	-0.042	-0.026	-0.209	-0.512
2.5		-1.891	-2.439	-1.023	-0.841	-0.308	-0.241

Standard errors of differences of means

Table	Area	Time_min	Treatment	Area Time_min
rep.	18	24	36	6
s.e.d.	0.2118	0.0959	0.0877	0.2634
d.f.	12	32	4	25.81
Except when comparing means with the same level(s) of				
Area				0.1917
d.f.				32

Table	Area Treatment	Time_min Treatment	Area Time_min Treatment
rep.	9	12	3
s.e.d.	0.2738	0.1412	0.3521
d.f.	14.34	20.44	32.91
Except when comparing means with the same level(s) of			
Treatment	0.2995	0.1356	0.3724
d.f.	12	32	25.81
Area.Treatment			0.2711
d.f.			32
Time_min.Treatment			0.3724
d.f.			25.81

Least significant differences of means (5% level)

Table	Area	Time_min	Treatment	Area Time_min
rep.	18	24	36	6
l.s.d.	0.4614	0.1953	0.2433	0.5415
d.f.	12	32	4	25.81
Except when comparing means with the same level(s) of				
Area				0.3905
d.f.				32

Table	Area Treatment	Time_min Treatment	Area Time_min Treatment
rep.	9	12	3
l.s.d.	0.5859	0.2941	0.7164
d.f.	14.34	20.44	32.91
Except when comparing means with the same level(s) of			
Treatment	0.6525	0.2762	0.7658
d.f.	12	32	25.81
Area.Treatment			0.5523
d.f.			32
Time_min.Treatment			0.7658
d.f.			25.81

3.3.2. Experiment with water-hatched *G. pallida* J2s

Analysis of variance

Variate: log_%_of_J2_1_101_%_of_J2 = log((%_of_J2+1)/(101-%_of_J2))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Dish stratum					
Treatment	1	0.0041	0.0041	0.01	0.934
Residual	4	2.0934	0.5233	0.52	
Dish.Area stratum					
Area	3	76.1555	25.3852	25.47	<.001
Area.Treatment	3	1.1557	0.3852	0.39	0.765
Residual	12	11.9622	0.9968	2.83	
Dish.Area.Time_min stratum					
Time_min	2	1.3076	0.6538	1.86	0.173
Area.Time_min	6	30.9283	5.1547	14.64	<.001
Time_min.Treatment	2	0.1899	0.0950	0.27	0.765
Area.Time_min.Treatment	6	2.7835	0.4639	1.32	0.278
Residual	32	11.2684	0.3521		
Total	71	137.8486			

Tables of means

Variate: log_%_of_J2_1_101_%_of_J2

Grand mean -1.461

Area	0.0	0.5	1.5	2.5
	-2.101	-1.087	0.023	-2.677

Time_min	30	60	90
	-1.650	-1.383	-1.349

Treatment	PRD (1:3)	Water
	-1.453	-1.468

Area	Time_min	30	60	90
0.0		-1.512	-2.037	-2.755
0.5		-0.683	-1.257	-1.320
1.5		-0.185	0.251	0.002
2.5		-4.222	-2.487	-1.324

Area	Treatment	PRD (1:3)	Water
0.0		-1.951	-2.251
0.5		-1.243	-0.930
1.5		0.133	-0.088
2.5		-2.751	-2.604

Time_min	Treatment	PRD (1:3)	Water
30		-1.714	-1.587
60		-1.328	-1.437
90		-1.318	-1.381

	Time_min	30		60		90	
Area	Treatment	PRD (1:3)	Water	PRD (1:3)	Water	PRD (1:3)	Water
0.0		-1.289	-1.734	-2.181	-1.893	-2.384	-3.126
0.5		-0.814	-0.553	-1.158	-1.356	-1.758	-0.881
1.5		-0.138	-0.231	0.301	0.200	0.236	-0.232
2.5		-4.615	-3.828	-2.272	-2.701	-1.364	-1.284

Standard errors of differences of means

Table	Area	Time_min	Treatment	Area Time_min
rep.	18	24	36	6
s.e.d.	0.3328	0.1713	0.1705	0.4348
d.f.	12	32	4	29.44
Except when comparing means with the same level(s) of				
Area				0.3426
d.f.				32

Table	Area Treatment	Time_min Treatment	Area Time_min Treatment
rep.	9	12	3
s.e.d.	0.4418	0.2612	0.5931
d.f.	15.17	17.95	37.75
Except when comparing means with the same level(s) of			
Treatment	0.4707	0.2423	0.6148
d.f.	12	32	29.44
Area.Treatment			0.4845
d.f.			32
Time_min.Treatment			0.6148
d.f.			29.44

Least significant differences of means (5% level)

Table	Area	Time_min	Treatment	Area Time_min
rep.	18	24	36	6
l.s.d.	0.7251	0.3489	0.4734	0.8886
d.f.	12	32	4	29.44
Except when comparing means with the same level(s) of				
Area				0.6979
d.f.				32

Table	Area Treatment	Time_min Treatment	Area Time_min Treatment
rep.	9	12	3
l.s.d.	0.9408	0.5488	1.2009
d.f.	15.17	17.95	37.75
Except when comparing means with the same level(s) of			
Treatment	1.0255	0.4935	1.2567
d.f.	12	32	29.44

Area.Treatment	0.9869
d.f.	32
Time_min.Treatment	
	1.2567
d.f.	29.44

3.3.3. Statistical analysis of combined data from 3.3.1 and 3.3.2

Analysis of variance

Variate: log_%_of_J2_1_101_%_of_J2 = log((%_of_J2+1)/(101-%_of_J2))

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Dish stratum					
Origin	1	0.0967	0.0967	0.33	0.583
Treatment	1	0.0000	0.0000	0.00	0.993
Origin.Treatment	1	0.0073	0.0073	0.02	0.879
Residual	8	2.3583	0.2948	0.47	
Dish.Area stratum					
Area	3	125.5962	41.8654	66.55	<.001
Area.Origin	3	32.0465	10.6822	16.98	<.001
Area.Treatment	3	0.4500	0.1500	0.24	0.869
Area.Origin.Treatment	3	0.9416	0.3139	0.50	0.687
Residual	24	15.0983	0.6291	2.82	
Dish.Area.Time_min stratum					
Time_min	2	0.5191	0.2595	1.16	0.319
Area.Time_min	6	43.5986	7.2664	32.61	<.001
Time_min.Origin	2	0.8500	0.4250	1.91	0.157
Time_min.Treatment	2	0.0341	0.0170	0.08	0.926
Area.Time_min.Origin	6	2.9491	0.4915	2.21	0.054
Area.Time_min.Treatment	6	2.2153	0.3692	1.66	0.146
Time_min.Origin.Treatment	2	0.3335	0.1667	0.75	0.477
Area.Time_min.Origin.Treatment	6	1.7138	0.2856	1.28	0.278
Residual	64	14.2596	0.2228		
Total	143	243.0681			

Tables of means

Variate: log_%_of_J2_1_101_%_of_J2

Grand mean -1.435

Area	0.0	0.5	1.5	2.5
	-2.586	-1.224	-0.049	-1.881

Time_min	30	60	90
	-1.519	-1.385	-1.400

Origin	PRD	Water
	-1.409	-1.461

Treatment	PRD (1:3)	Water
	-1.434	-1.435

Area	Time_min	30	60	90
0.0		-2.154	-2.587	-3.015
0.5		-0.695	-1.365	-1.611
1.5		-0.080	0.109	-0.176
2.5		-3.148	-1.699	-0.797

Area	Origin	PRD	Water
0.0		-3.070	-2.101
0.5		-1.361	-1.087
1.5		-0.120	0.023
2.5		-1.085	-2.677

Time_min	Origin	PRD	Water
30		-1.388	-1.650
60		-1.388	-1.383
90		-1.450	-1.349

Area	Treatment	PRD (1:3)	Water
0.0		-2.561	-2.610
0.5		-1.304	-1.144
1.5		0.024	-0.122
2.5		-1.897	-1.866

Time_min	Treatment	PRD (1:3)	Water
30		-1.523	-1.516
60		-1.365	-1.406
90		-1.416	-1.384

Origin	Treatment	PRD (1:3)	Water
PRD		-1.416	-1.402
Water		-1.453	-1.468

	Time_min	30		60		90	
Area	Origin	PRD	Water	PRD	Water	PRD	Water
0.0		-2.797	-1.512	-3.138	-2.037	-3.276	-2.755
0.5		-0.707	-0.683	-1.472	-1.257	-1.903	-1.320
1.5		0.025	-0.185	-0.033	0.251	-0.353	0.002
2.5		-2.074	-4.222	-0.910	-2.487	-0.269	-1.324

	Time_min	30		60		90	
Area	Treatment	PRD (1:3)	Water	PRD (1:3)	Water	PRD (1:3)	Water
0.0		-1.994	-2.314	-2.746	-2.428	-2.944	-3.087
0.5		-0.802	-0.589	-1.206	-1.523	-1.903	-1.320
1.5		-0.074	-0.086	0.130	0.088	0.016	-0.367
2.5		-3.221	-3.075	-1.636	-1.762	-0.833	-0.760

	Origin	PRD		Water	
Area	Treatment	PRD (1:3)	Water	PRD (1:3)	Water
0.0		-3.171	-2.969	-1.951	-2.251
0.5		-1.364	-1.358	-1.243	-0.930
1.5		-0.085	-0.156	0.133	-0.088
2.5		-1.042	-1.127	-2.751	-2.604

	Origin	PRD		Water	
Time_min	Treatment	PRD (1:3)	Water	PRD (1:3)	Water
30		-1.331	-1.445	-1.714	-1.587
60		-1.401	-1.375	-1.328	-1.437
90		-1.514	-1.386	-1.318	-1.381

		Origin	PRD		Water	
Area	Time_min	Treatment	PRD (1:3)	Water	PRD (1:3)	Water
0.0	30		-2.699	-2.895	-1.289	-1.734
	60		-3.311	-2.964	-2.181	-1.893
	90		-3.503	-3.048	-2.384	-3.126
0.5	30		-0.790	-0.624	-0.814	-0.553
	60		-1.254	-1.689	-1.158	-1.356
	90		-2.047	-1.759	-1.758	-0.881
1.5	30		-0.009	0.060	-0.138	-0.231
	60		-0.041	-0.025	0.301	0.200
	90		-0.205	-0.502	0.236	-0.232
2.5	30		-1.826	-2.322	-4.615	-3.828
	60		-0.999	-0.822	-2.272	-2.701
	90		-0.302	-0.236	-1.364	-1.284

Standard errors of differences of means

Table	Area	Time_min	Origin	Treatment
rep.	36	48	72	72
d.f.	24	64	8	8
s.e.d.	0.1869	0.0964	0.0905	0.0905

Table	Area Time_min	Area Origin	Time_min Origin	Area Treatment
rep.	12	18	24	18
s.e.d.	0.2443	0.2462	0.1434	0.2462
d.f.	58.95	29.89	39.25	29.89
Except when comparing means with the same level(s) of				
Area	0.1927			
d.f.	64			
Origin		0.2644	0.1363	
d.f.		24	64	
Treatment				0.2644
d.f.				24

Table	Time_min Treatment	Origin Treatment	Area Time_min Origin	Area Time_min Treatment
rep.	24	36	6	6
s.e.d.	0.1434	0.1280	0.3319	0.3319
d.f.	39.25	8	75.23	75.23
Except when comparing means with the same level(s) of				
Origin			0.3456	
d.f.			58.95	
Treatment	0.1363			0.3456
d.f.	64			58.95
Area.Origin			0.2725	
d.f.			64	
Time_min.Origin			0.3456	
d.f.			58.95	
Area.Treatment				0.2725
d.f.				64
Time_min.Treatment				0.3456
d.f.				58.95

Table	Area Origin Treatment	Time_min Origin Treatment	Area Time_min Origin Treatment
rep.	9	12	3
s.e.d.	0.3482	0.2028	0.4693
d.f.	29.89	39.25	75.23
Except when comparing means with the same level(s) of Origin.Treatment	0.3739	0.1927	0.4887
d.f.	24	64	58.95
Area.Origin.Treatment			0.3854
d.f.			64
Time_min.Origin.Treatment			0.4887
d.f.			58.95

Least significant differences of means (5% level)

Table	Area	Time_min	Origin	Treatment
rep.	36	48	72	72
d.f.	24	64	8	8
l.s.d.	0.3858	0.1925	0.2087	0.2087

Table	Area Time_min	Area Origin	Time_min Origin	Area Treatment
rep.	12	18	24	18
l.s.d.	0.4889	0.5029	0.2900	0.5029
d.f.	58.95	29.89	39.25	29.89
Except when comparing means with the same level(s) of				
Area	0.3850			
d.f.	64			
Origin		0.5457	0.2722	
d.f.		24	64	
Treatment				0.5457
d.f.				24

Table	Time_min Treatment	Origin Treatment	Area Time_min Origin	Area Time_min Treatment
rep.	24	36	6	6
l.s.d.	0.2900	0.2951	0.6611	0.6611
d.f.	39.25	8	75.23	75.23
Except when comparing means with the same level(s) of				
Origin			0.6915	
d.f.			58.95	
Treatment	0.2722			0.6915
d.f.	64			58.95
Area.Origin			0.5444	
d.f.			64	
Time_min.Origin			0.6915	
d.f.			58.95	
Area.Treatment				0.5444
d.f.				64
Time_min.Treatment				0.6915
d.f.				58.95

Table	Area Origin Treatment	Time_min Origin Treatment	Area Time_min Origin Treatment
rep.	9	12	3
l.s.d.	0.7112	0.4101	0.9349
d.f.	29.89	39.25	75.23
Except when comparing means with the same level(s) of Origin.Treatment			
	0.7717	0.3850	0.9779
d.f.	24	64	58.95
Area.Origin.Treatment			0.7699
d.f.			64
Time_min.Origin.Treatment			0.9779
d.f.			58.95

3.4. Invasion and development of *G. pallida* in *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

3.4.1. Invasion bioassays

Analysis of variance

Variate: LOG_No_of_J2s_per_gram_root

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Plant_species	1	9.0025	9.0025	67.46	<.001
dpi	3	3.3169	1.1056	8.28	0.001
Plant_species.dpi	3	1.3250	0.4417	3.31	0.047
Residual	16	2.1353	0.1335		
Total	23				

Tables of means

Variate: LOG_No_of_J2s_per_gram_root

Grand mean 6.310

Plant_species	Ss	St
	6.923	5.698

dpi	1	2	3	4
	6.042	5.868	6.535	6.795

Plant_species	dpi	1	2	3	4
Ss		6.372	6.681	7.409	7.228
St		5.712	5.056	5.661	6.363

Standard errors of differences of means

Table	Plant_species	dpi	Plant_species dpi
rep.	12	6	3
d.f.	16	16	16
s.e.d.	0.1491	0.2109	0.2983

Least significant differences of means (5% level)

Table	Plant_species	dpi	Plant_species dpi
rep.	12	6	3
d.f.	16	16	16
l.s.d.	0.3162	0.4471	0.6323

5.2. Comparison of defence-related gene expression changes in the time course infection with *G. pallida* between *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*: qRT-PCR analysis

Solanum tuberosum L. cv. Desiree

ACRE

Analysis of variance

Variate: Ctacre_Ct18S

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample stratum					
Treatment	2	0.50688	0.25344	0.76	0.507
Residual	6	1.99347	0.33224	7.23	
Sample.Technical_repeat stratum	9	0.41335	0.04593		
Total	17	2.91369			

Tables of means

Variate: Ctacre_Ct18S

Grand mean 12.599

Treatment	10 dpi	3 dpi	Uninfected
	12.790	12.627	12.382

Standard errors of differences of means

Table	Treatment
rep.	6
d.f.	6
s.e.d.	0.3328

Least significant differences of means (5% level)

Table	Treatment
rep.	6
d.f.	6
l.s.d.	0.8143

PAL

Analysis of variance

Variate: CtPAL_Ct18S

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample stratum					
Treatment	2	26.73043	13.36522	22.53	0.002
Residual	6	3.55867	0.59311	14.50	
Sample.Technical_repeat stratum					
	9	0.36815	0.04091		
Total	17	30.65725			

Tables of means

Variate: CtPAL_Ct18S

Grand mean 6.645

Treatment	10 dpi	3 dpi	Uninfected
	5.418	6.210	8.307

Standard errors of differences of means

Table	Treatment
rep.	6
d.f.	6
s.e.d.	0.4446

Least significant differences of means (5% level)

Table	Treatment
rep.	6
d.f.	6
l.s.d.	1.0880

Chitinase

Analysis of variance

Variate: Ctcht_Ct18S

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample stratum					
Treatment	2	4.96023	2.48012	7.26	0.025
Residual	6	2.05027	0.34171	5.45	
Sample.Technical_repeat stratum					
	9	0.56390	0.06266		
Total	17	7.57440			

Tables of means

Variate: Ctcht_Ct18S

Grand mean 7.770

Treatment	10 dpi	3 dpi	Uninfected
	7.185	7.667	8.458

Standard errors of differences of means

Table	Treatment
rep.	6
d.f.	6
s.e.d.	0.3375

Least significant differences of means (5% level)

Table	Treatment
rep.	6
d.f.	6
l.s.d.	0.8258

Solanum sisymbriifolium

ACRE

Analysis of variance

Variate: Ctacre_Ct18S

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample stratum					
Treatment	2	1.5358	0.7679	1.83	0.239
Residual	6	2.5154	0.4192	2.91	
Sample.Treatment.*Units* stratum					
	9	1.2979	0.1442		
Total	17	5.3492			

Tables of means

Variate: Ctacre_Ct18S

Grand mean 12.893

Treatment	10 dpi	3 dpi	Uninfected
	13.273	12.563	12.842

Standard errors of differences of means

Table	Treatment
rep.	6
d.f.	6
s.e.d.	0.3738

Least significant differences of means (5% level)

Table	Treatment
rep.	6
d.f.	6
l.s.d.	0.9147

PAL

Analysis of variance

Variate: CtPAL_Ct18S

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample stratum					
Treatment	2	22.6147	11.3074	8.61	0.017
Residual	6	7.8787	1.3131	3.22	
Sample.Treatment.*Units* stratum	9	3.6663	0.4074		
Total	17	34.1598			

Tables of means

Variate: CtPAL_Ct18S

Grand mean 17.58

Treatment	10 dpi	3 dpi	Uninfected
	17.02	16.58	19.15

Standard errors of differences of means

Table	Treatment
rep.	6
d.f.	6
s.e.d.	0.662

Least significant differences of means (5% level)

Table	Treatment
rep.	6
d.f.	6
l.s.d.	1.619

Chitinase

Analysis of variance

Variate: Ctcht_Ct18S

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sample stratum					
Treatment	2	19.66813	9.83407	11.17	0.009
Residual	6	5.28457	0.88076	21.87	
Sample.Treatment.*Units* stratum	9	0.36250	0.04028		
Total	17	25.31520			

Tables of means

Variate: Ctcht_Ct18S

Grand mean 8.773

Treatment	10 dpi	3 dpi	Uninfected
	7.743	8.370	10.207

Standard errors of differences of means

Table	Treatment
rep.	6
d.f.	6
s.e.d.	0.5418

Least significant differences of means (5% level)

Table	Treatment
rep.	6
d.f.	6
l.s.d.	1.3258

6.1. Hatching ability of root exudate extracts

6.1.2. Investigation of a Hatching Factor (HF) of *S. sisymbriifolium*

6.1.2.1. Determining the optimum concentration of the root exudate extracts from *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium* to induce hatching of *G. pallida* J2s

Analysis of variance

Variate: SQRT_%_of_hatch

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Plant_Species	1	0.40523	0.40523	4.19	0.050
Concentrations	6	23.94487	3.99081	41.24	<.001
Plant_Species.Concentrations	6	0.61097	0.10183	1.05	0.414
Residual	28	2.70972	0.09678		
Total	41	27.67078			

Tables of means

Variate: SQRT_%_of_hatch

Grand mean 1.394

Plant_Species	S.s.	S.t.
	1.296	1.492

Concentrations	1x10	1x102	1x103	1x104	1x105	1x106
	2.386	1.312	1.072	1.113	0.648	0.573

Concentrations	Undiluted
	2.654

Plant_Species	Concentrations	1x10	1x102	1x103	1x104	1x105
S.s.		2.395	1.064	1.020	0.829	0.694
S.t.		2.377	1.559	1.124	1.398	0.603

Plant_Species	Concentrations	1x106	Undiluted
S.s.		0.424	2.645
S.t.		0.722	2.663

Standard errors of differences of means

Table	Plant_Species	Concentrations	Plant_Species Concentrations
rep.	21	6	3
d.f.	28	28	28
s.e.d.	0.0960	0.1796	0.2540

Least significant differences of means (5% level)

Table	Plant_Species	Concentrations	Plant_Species Concentrations
rep.	21	6	3
d.f.	28	28	28
l.s.d.	0.1967	0.3679	0.5203

6.1.2.2. Comparison of hatching ability of reversed-phase HPLC fractions from root exudate extracts of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

Analysis of variance

Variate: SQRT(%_of_hatch)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Plant_species	1	0.5118	0.5118	1.87	0.184
Fraction	5	3.0686	0.6137	2.25	0.082
Plant_species.Fraction	5	6.4874	1.2975	4.75	0.004
Residual	24	6.5511	0.2730		
Total	35	16.6189			

Tables of means

Variate: SQRT(%_of_hatch)

Grand mean 1.388

Plant_species	S.s.	S.t.
	1.268	1.507

Fraction	A	B	C	D	E	Unfractionated
	1.156	1.284	1.506	1.023	1.422	1.934

Plant_species	Fraction	A	B	C	D	E	Unfractionated
S.s.		0.566	1.987	0.964	0.896	1.310	1.886
S.t.		1.746	0.581	2.049	1.149	1.534	1.982

Standard errors of differences of means

Table	Plant_species	Fraction	Plant_species Fraction
rep.	18	6	3
d.f.	24	24	24
s.e.d.	0.1742	0.3016	0.4266

Least significant differences of means (5% level)

Table	Plant_species	Fraction	Plant_species Fraction
rep.	18	6	3
d.f.	24	24	24
l.s.d.	0.3594	0.6226	0.8804

6.1.2.3. Bioassay-guided reversed-phase HPLC sub-fractionation of root exudate extract of *S. sisymbriifolium*

Hatching assay with sub-fractions of fraction B from* S. sisymbriifolium *root exudate extract

Analysis of variance

Variate: SQRT_%_hatch

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sub_fraction	3	8.7641	2.9214	25.11	<.001
Residual	8	0.9309	0.1164		
Total	11	9.6950			

Tables of means

Variate: SQRT_%_hatch

Grand mean 1.323

Sub_fraction	B1	B2
	2.340	0.528

Sub_fraction	B3	Unfractionated
	0.428	1.996

Standard errors of differences of means

Table	Sub_fraction
rep.	3
d.f.	8
s.e.d.	0.2785

Least significant differences of means (5% level)

Table	Sub_fraction
rep.	3
d.f.	8
l.s.d.	0.6423

Hatching assay with sub-fractions of fraction B1 from* S. sisymbriifolium *root exudate extract

Analysis of variance

Variate: SQRT_%_hatch

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Sub_fraction	5	13.6297	2.7259	6.31	0.004
Residual	12	5.1810	0.4317		
Total	17	18.8106			

Tables of means

Variate: SQRT_%_hatch

Grand mean 1.87

Sub_fraction	B1-a	B1-b
	1.23	1.53
Sub_fraction	B1-c	B1-d
	1.52	3.23
Sub_fraction	B1-e	B1
	0.84	2.85

Standard errors of differences of means

Table	Sub_fraction
rep.	3
d.f.	12
s.e.d.	0.536

Least significant differences of means (5% level)

Table	Sub_fraction
rep.	3
d.f.	12
l.s.d.	1.169

6.2. Hatching ability of aerial part extract

6.2.1. Ethanol extract from aerial part of *S. sisymbriifolium*

Analysis of variance

Variate: SQRT_No_of_J2_hatched

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
TW_control	1	23.945	23.945	11.74	0.002
TW_control.PRD_control	1	120.851	120.851	59.23	<.001
TW_control.PRD_control.Treatment	1	0.034	0.034	0.02	0.899
TW_control.PRD_control.Concentration	4	25.844	6.461	3.17	0.032
TW_control.PRD_control.Treatment.Concentration	4	11.038	2.759	1.35	0.280
Residual	24	48.971	2.040		
Total	35	230.683			

Tables of means

Variate: SQRT_No_of_J2_hatched

Grand mean 3.04

TW_control	Rest 1	TW
	3.28	0.33
rep.	33	3

TW_control	PRD_control	PRD	Rest 2	TW
Rest 1		9.34	2.68	
	rep.	3	30	
TW				0.33
	rep.			3

TW_control	PRD_control	Treatment	EtOH	Extract	PRD	TW
Rest 1	PRD				9.34	
		rep.			3	
	Rest 2		2.65	2.71		
		rep.	15	15		
TW	TW					0.33
		rep.				3

TW_control	PRD_control	Concentration	10-fold dilution	100-fold dilution
Rest 1	Rest 2		2.70	2.19
		rep.	6	6

TW_control	PRD_control	Concentration	5-fold dilution	50-fold dilution
Rest 1	Rest 2		4.42	2.37
		rep.	6	6

TW_control	PRD_control	Concentration	PRD	TW
Rest 1	PRD		9.34	
		rep.	3	
TW	TW			0.33
		rep.		3

TW_control	PRD_control	Concentration	Undiluted
Rest 1	Rest 2		1.71
		rep.	6

TW_control	PRD_control	Treatment	Concentration	10-fold dilution
Rest 1	Rest 2	EtOH		3.02
		Extract		2.37

TW_control	PRD_control	Treatment	Concentration	100-fold dilution
Rest 1	Rest 2	EtOH		2.38
		Extract		2.00

TW_control	PRD_control	Treatment	Concentration	5-fold dilution
Rest 1	Rest 2	EtOH		3.32
		Extract		5.52

TW_control	PRD_control	Treatment	Concentration	50-fold dilution
Rest 1	Rest 2	EtOH		3.03
		Extract		1.71

TW_control	PRD_control	Treatment	Concentration	PRD
Rest 1	PRD	PRD		9.34

TW_control	PRD_control	Treatment	Concentration	TW
TW	TW	TW		0.33

TW_control	PRD_control	Treatment	Concentration	Undiluted
Rest 1	Rest 2	EtOH		1.47
		Extract		1.96

Standard errors of differences of means

Table	TW_control	TW_control PRD_control	TW_control PRD_control Treatment	TW_control PRD_control Concentration	
rep.	unequal	unequal	unequal	unequal	
d.f.	24	24	24	24	
s.e.d.		1.166	1.166	1.166	min.rep
	0.861	0.865	0.903	1.010	max-min
		0.369X	0.522	0.825	max.rep

Table	TW_control PRD_control Treatment Concentration
rep.	3
d.f.	24
s.e.d.	1.166

(No comparisons in categories where s.e.d. marked with an X)

Least significant differences of means (5% level)

Table	TW_control	TW_control PRD_control	TW_control PRD_control Treatment	TW_control PRD_control Concentration	
rep.	unequal	unequal	unequal	unequal	
d.f.	24	24	24	24	
l.s.d.		2.407	2.407	2.407	min.rep
	1.778	1.785	1.865	2.085	max-min
		0.761X	1.077	1.702	max.rep

Table	TW_control PRD_control Treatment Concentration
rep.	3
d.f.	24
l.s.d.	2.407

(No comparisons in categories where l.s.d. marked with an X)

6.2.2. Aqueous extract from macerated aerial part of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

Analysis of variance

Variate: SQRT_%_hatch

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
DW_control	1	22.067	22.067	21.60	<.001
DW_control.PRD_control	1	26.388	26.388	25.83	<.001
DW_control.PRD_control.Plant_species	1	1.119	1.119	1.09	0.306
DW_control.PRD_control.Dose	4	18.033	4.508	4.41	0.008
DW_control.PRD_control.Plant_species.Dose	4	17.697	4.424	4.33	0.009
Residual	24	24.519	1.022		
Total	35	109.823			

Tables of means

Variate: SQRT_%_hatch

Grand mean 2.60

DW_control	DW	Rest 1
	0.00	2.83
rep.	3	33

DW_control	PRD_control	DW	PRD	Rest 2
DW		0.00		
	rep.	3		
Rest 1			5.66	2.55
	rep.		3	30

DW_control	PRD_control	Plant_species	DW	PRD	Ss	St
DW	DW		0.00			
		rep.	3			
Rest 1	PRD			5.66		
		rep.		3		
	Rest 2				2.74	2.36
		rep.			15	15

DW_control	PRD_control	Dose	1 mg/ml	10 mg/ml	100 mg/ml	2 mg/ml
Rest 1	Rest 2		2.99	3.13	1.17	3.24
		rep.	6	6	6	6

DW_control	PRD_control	Dose	20 mg/ml	DW	PRD
DW	DW			0.00	
		rep.		3	
Rest 1	PRD				5.66
		rep.			3
	Rest 2		2.22		
		rep.	6		

DW_control	PRD_control	Plant_species	Dose	1 mg/ml	10 mg/ml
Rest 1	Rest 2	Ss		3.02	4.45
		St		2.97	1.81

DW_control	PRD_control	Plant_species	Dose	100 mg/ml	2 mg/ml
Rest 1	Rest 2	Ss		0.23	3.93
		St		2.12	2.54

DW_control	PRD_control	Plant_species	Dose	20 mg/ml	DW
DW	DW	DW			0.00
Rest 1	Rest 2	Ss		2.08	
		St		2.36	

DW_control	PRD_control	Plant_species	Dose	PRD
Rest 1	PRD	PRD		5.66

Standard errors of differences of means

Table	DW_control	DW_control PRD_control	DW_control PRD_control Plant_species	DW_control PRD_control Dose	
rep.	unequal	unequal	unequal	unequal	
d.f.	24	24	24	24	
s.e.d.		0.825	0.825	0.825	min.rep
	0.610	0.612	0.639	0.715	max-min
		0.261X	0.369	0.584	max.rep

Table	DW_control PRD_control Plant_species Dose
rep.	3
d.f.	24
s.e.d.	0.825

(No comparisons in categories where s.e.d. marked with an X)

Least significant differences of means (5% level)

Table	DW_control	DW_control PRD_control	DW_control PRD_control Plant_species	DW_control PRD_control Dose	
rep.	unequal	unequal	unequal	unequal	
d.f.	24	24	24	24	
l.s.d.		1.703	1.703	1.703	min.rep
	1.258	1.263	1.319	1.475	max-min
		0.539X	0.762	1.204	max.rep

Table	DW_control PRD_control Plant_species Dose
rep.	3
d.f.	24
l.s.d.	1.703

(No comparisons in categories where l.s.d. marked with an X)

6.2.3. Aqueous extract from intact aerial part of *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

Analysis of variance

Variate: SQRT_%_hatch

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
DW_control	1	20.9525	20.9525	30.73	<.001
DW_control.PRD_control	1	8.5143	8.5143	12.49	0.002
DW_control.PRD_control.Plant_species	1	26.1714	26.1714	38.38	<.001
DW_control.PRD_control.Concentration	4	24.2897	6.0724	8.91	<.001
DW_control.PRD_control.Plant_species.Concentration	4	10.3201	2.5800	3.78	0.016
Residual	24	16.3646	0.6819		
Total	35	106.6126			

Tables of means

Variate: SQRT_%_hatch

Grand mean 2.79

DW_control	DW	Rest 1
	0.26	3.02
rep.	3	33

DW_control	PRD_control	DW	PRD	Rest 2
DW		0.26		
	rep.	3		
Rest 1			4.62	2.85
	rep.		3	30

DW_control	PRD_control	Plant_species	DW	PRD	Ss	St
DW	DW		0.26			
		rep.	3			
Rest 1	PRD			4.62		
		rep.		3		
	Rest 2				3.79	1.92
		rep.			15	15

DW_control	PRD_control	Concentration	0.01 mg/ml	0.02 mg/ml	0.1 mg/ml
Rest 1	Rest 2		1.38	2.28	3.49
		rep.	6	6	6

DW_control	PRD_control	Concentration	0.2 mg/ml	1 mg/ml	DW
DW	DW				0.26
		rep.			3
Rest 1	Rest 2		3.84	3.28	
		rep.	6	6	

DW_control	PRD_control	Concentration	PRD
Rest 1	PRD		4.62
		rep.	3

DW_control	PRD_control	Plant_species	Concentration	0.01 mg/ml	0.02 mg/ml
Rest 1	Rest 2	Ss		1.57	2.84
		St		1.20	1.72

DW_control	PRD_control	Plant_species	Concentration	0.1 mg/ml	0.2 mg/ml
Rest 1	Rest 2	Ss		4.64	4.70
		St		2.34	2.98

DW_control	PRD_control	Plant_species	Concentration	1 mg/ml	DW
DW	DW	DW			0.26
Rest 1	Rest 2	Ss		5.20	
		St		1.36	

DW_control	PRD_control	Plant_species	Concentration	PRD
Rest 1	PRD	PRD		4.62

Standard errors of differences of means

Table	DW_control	DW_control PRD_control	DW_control PRD_control Plant_species	DW_control PRD_control Concentration	
rep.	unequal	unequal	unequal	unequal	
d.f.	24	24	24	24	
s.e.d.		0.674	0.674	0.674	min.rep
	0.498	0.500	0.522	0.584	max-min
		0.213X	0.302	0.477	max.rep

Table	DW_control PRD_control Plant_species Concentration
rep.	3
d.f.	24
s.e.d.	0.674

(No comparisons in categories where s.e.d. marked with an X)

Least significant differences of means (5% level)

Table	DW_control	DW_control PRD_control	DW_control PRD_control Plant_species	DW_control PRD_control Concentration	
rep.	unequal	unequal	unequal	unequal	
d.f.	24	24	24	24	
l.s.d.		1.392	1.392	1.392	min.rep
	1.028	1.032	1.078	1.205	max-min
		0.440X	0.622	0.984	max.rep

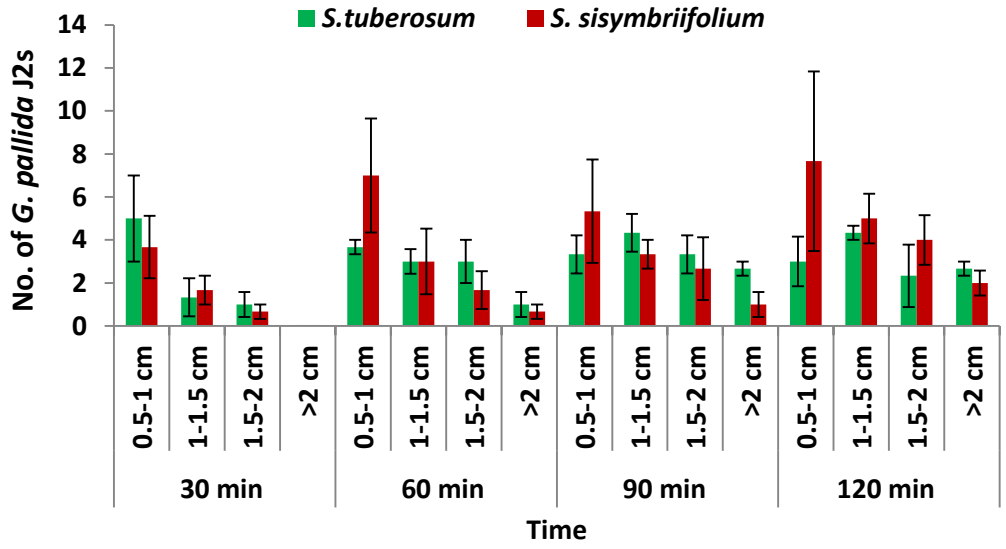
Table	DW_control PRD_control Plant_species Concentration
rep.	3
d.f.	24
l.s.d.	1.392

(No comparisons in categories where l.s.d. marked with an X)

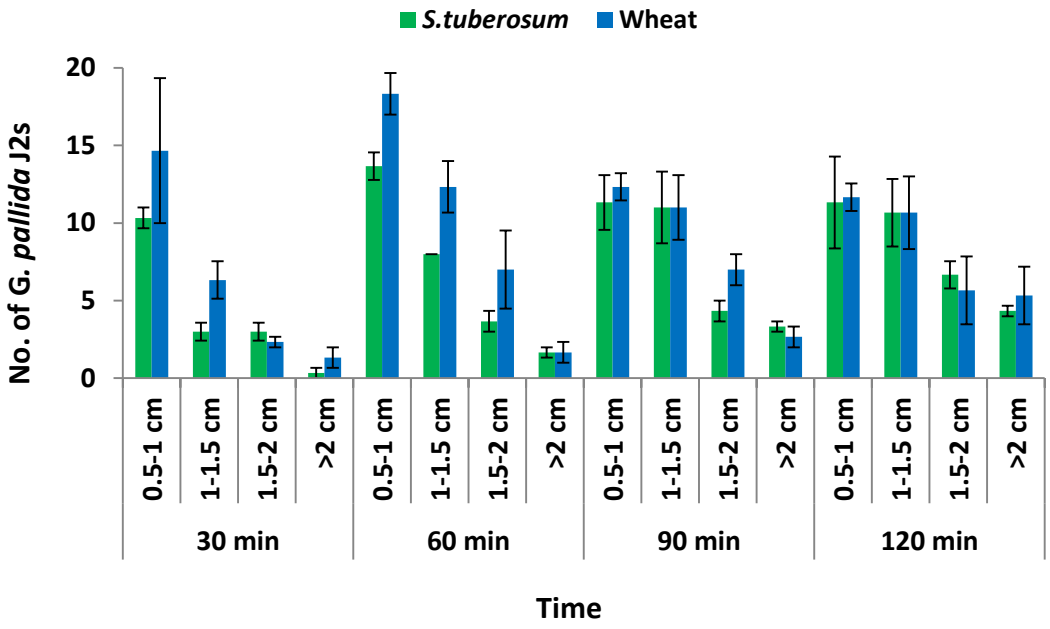
Appendix II: Raw data means and standard errors

3.1.2. Choice assays between two plant species (attraction assays)

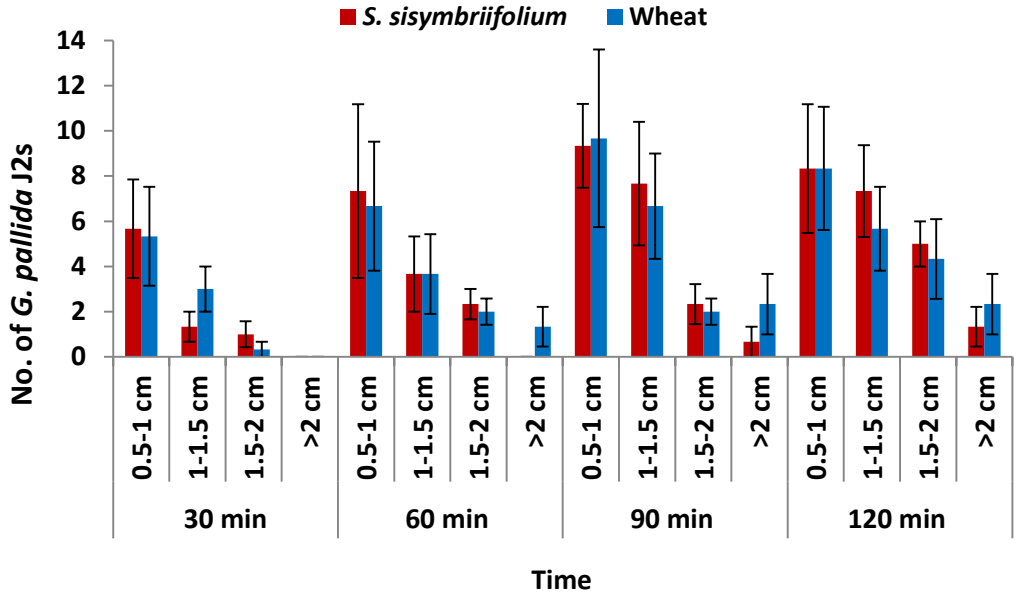
3.1.2.1. Between *S. tuberosum* L. cv. Desiree (host) and *S. sisymbriifolium* (trap crop)



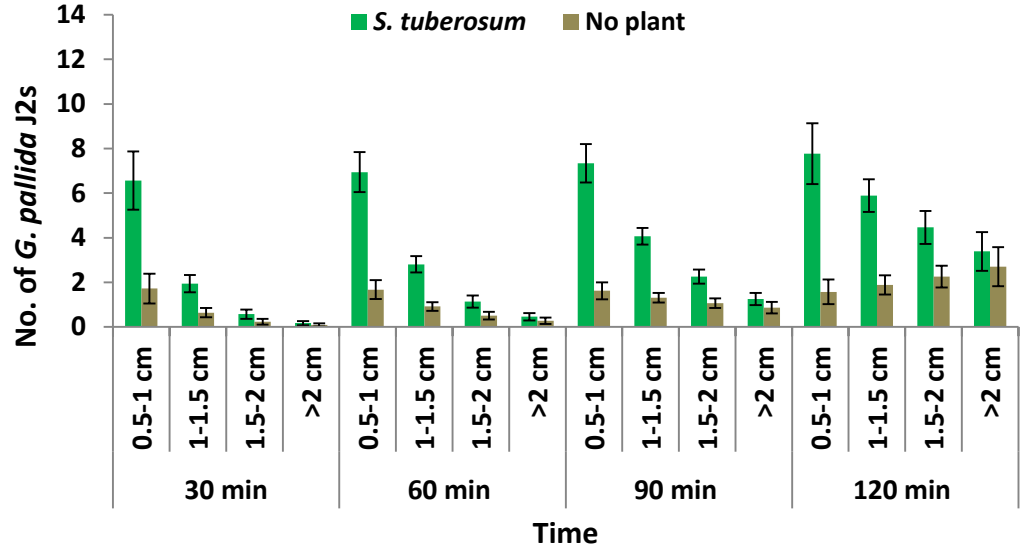
3.1.2.2. Between *S. tuberosum* L. cv. Desiree (host) and wheat (non-host)



3.1.2.3. Between *S. sisymbriifolium* (trap crop) and wheat (non-host)

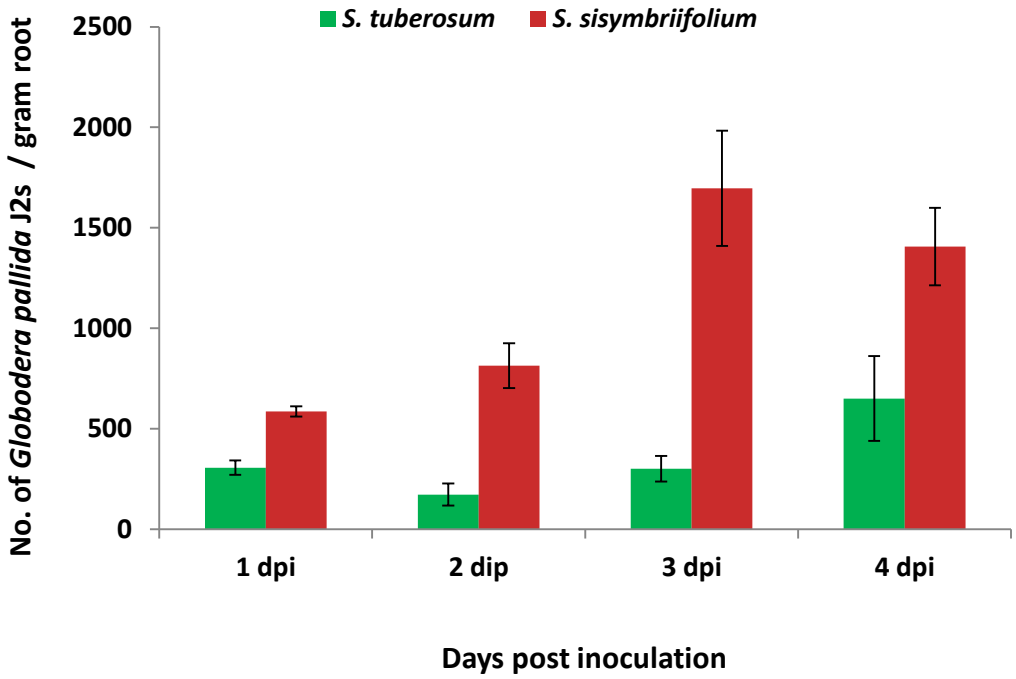


3.1.2.4. Between *S. tuberosum* L. cv. Desiree (host) and “no plant”



3.4. Invasion and development of *G. pallida* in *S. tuberosum* L. cv. Desiree and *S. sisymbriifolium*

3.4.1. Invasion bioassays



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Signalling and behaviour of potato cyst nematode in the rhizosphere of the trap crop, *Solanum sisymbriifolium*

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Summary

Potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, are commercially important pests of potato crops in the UK and mainland Europe. The non-tuber-bearing *Solanum* species, *S. sisymbriifolium*, has been used as an effective trap crop, but its mode of action is undefined. The interaction between *G. pallida* and *S. sisymbriifolium* has been investigated through attraction and invasion/development bioassays. *Globodera pallida* second-stage juveniles were attracted to the roots of *S. sisymbriifolium* in a similar way to those of *S. tuberosum*, with the invasion per gram of root exceeding that for *S. tuberosum*. However, time course observation studies indicated that the nematodes in *S. sisymbriifolium* roots failed to follow the normal life cycle seen in *S. tuberosum*. Studies of root exudate extracts of the two *Solanum* species so far suggest that the hatching factor of *S. sisymbriifolium* differs from that of *S. tuberosum*, with solanoeclepin A not detected.

Key words: Potato cyst nematode (PCN), *Globodera rostochiensis*, *Globodera pallida*, trap crop, *Solanum sisymbriifolium*, *Solanum tuberosum*, solanoeclepin A

Introduction

Trap cropping is an alternative strategy able to complement other integrated control measures against potato cyst nematodes (PCN), and is of increasing importance due to restrictions on the use of nematicides in Europe (European Union Directive 91/414/EEC). Another issue in the control of PCN is the recent change of dominance from *G. rostochiensis* (Woll.) to *G. pallida* (Stone) in England and Wales, partly due to excessive use of cultivars resistant only to *G. rostochiensis* (Minnis *et al*., 2002). *Globodera pallida* is

more difficult to control due to: a) more heterogeneous and varied pathotypes (Folkertsma *et al*., 1996), b) relatively slow hatch (Whitehead, 1992), and c) lower population decline rates (Minnis *et al*., 2002), than *G. rostochiensis*.

Scholte (2000*b*) demonstrated that a non-tuber-bearing *Solanum* species, *S. sisymbriifolium* Lamarck (Solanaceae), could be an effective trap crop with strong hatch stimulation and total resistance to both *G. rostochiensis* and *G. pallida*. It is particularly important that a trap crop is non-tuber-bearing, because it poses no danger of producing volunteer plants in the following year (Scholte, 2000*a*). Furthermore, it can be ploughed in as green manure (Timmermans, 2005) instead of removing it mechanically or with systemic herbicides. *Solanum sisymbriifolium* has been used as an effective trap crop in the UK and the Netherlands, but the mode of action has not been defined. It was shown that PCN second-stage juveniles (J2s) invaded roots in fairly large numbers (Roberts & Stone, 1983) and yet no cysts developed (Roberts & Stone, 1983; Scholte, 2000*b*; Yamada *et al*., 2007). However, there has been no report on how strongly PCN J2s are attracted to the roots of *S. sisymbriifolium* and how quickly and readily they invade them, compared with potato, *S. tuberosum* Linnaeus, 1753.

Also, no investigation has so far been conducted into a hatching factor (HF) present in *S. sisymbriifolium* that induces PCN J2s to hatch. For *S. tuberosum*, the presence of at least nine or 10 HFs has been reported (Devine *et al*., 1996; Devine & Jones, 2000), but so far only one HF has been identified, named solanoeclepin A, with the empirical formula $C_{27}H_{30}O_9$ (Mulder *et al*., 1996).

Our aim is to have a better understanding of the interaction and signalling between *S. sisymbriifolium* and *G. pallida*, so that a novel nematode control strategy could be developed.

Materials and Methods

Attraction bioassays

Plant materials

Seedlings of *S. sisymbriifolium* (Branston Ltd) were grown at *circa* (*c.*) 25°C on Gamborg's B5 Basal Medium with Minimal Organics (Sigma) with 0.15% sucrose (Fisher Scientific), 0.8% agar (Sigma) and Gamborg's Vitamin Solution 1000 × (Sigma), pH 6.4. Plantlets of *S. tuberosum* L. cv. Desirée were grown at *c*. 20°C from small sprouts removed from tubers by soaking them in water. The plants used in the bioassay experiments will be referred as plantlets and they were *c*. 5-days-old after root emergence.

Nematodes (Globodera pallida)

The population used was cultured on *S. tuberosum* L. cv. Maris Piper in a tub with soil collected from a *G. pallida*-infested field in Waddington, Lincolnshire, UK. The cysts were soaked in water for 3–5 days, and then the water was replaced with potato root diffusate (PRD) diluted four times with water and left at *c*. 18°C for J2s to hatch (Clarke & Perry, 1977). Hatched J2s were collected every 24–48 h, centrifuged at 2500 rpm for 5 minutes after being cooled to *c*. 4°C for 30 min, and used immediately.

Assay

Approximately 100 J2s, suspended in a minimum volume of 0.1 M phosphate buffered saline (PBS: sodium chloride (BDH), sodium dihydrogen phosphate dehydrate (Fisher

Scientific), disodium phosphate dodecahydrate (Fisher Scientific), pH 7.2) were placed in a 3.5 cm-diameter Petri dish. One mL of Pluronic F-127 (Sigma) (2.3 g in 10 mL of distilled water) was added and then mixed with the J2s evenly. Before the gel set, a plantlet was placed in the centre, and the number of the J2s in "the attraction zone" (within 3 mm of the root surface) was counted immediately, and this constituted an observation at time zero. One replicate of the experiment consisted of four Petri dishes for: *S. sisymbriifolium*, *S. tuberosum* and their corresponding controls. A control had no plant material, but with the shape of its corresponding plant species outlined on the lid of the Petri dish. The number of the J2s in the attraction zone was counted at 0, 30, 60 and 120 min, using a Vickers Instruments stereo dissecting microscope. The experiment was replicated five times. Proportions of the J2s that were in the attraction zone at the four time points were calculated for each of the dishes in each experimental replicate. For statistical analysis, split-plot in time ANOVA was implemented for five blocks (this being the replication), using natural log of the plant-to-control ratio. Relevant means were compared using Least Significant Difference (LSD) at the 5% level of significance. GenStat® (2009, twelfth Edition, © Lawes Agricultural Trust (Rothamsted Research), VSN International Ltd, Hemel Hempstead, UK) was used.

Invasion/development assays

Plant materials

After emergence, plantlets of *S. sisymbriifolium* and *S. tuberosum* L. cv. Desirée were planted individually in 6 cm × 6 cm pots with weed mix (80% sterilised Screened Loam, 20% Lime Free Grit (3–6 mm), 2 kg m^{-3} Osmocote) and grown at 20°C with 16:8 hours light:dark. Plantlets of *S. sisymbriifolium* were produced by sowing seeds in a small multi-cell seed tray with weed mix, whereas *S. tuberosum* were grown from small sprouts detached from tubers.

Assay

Two to three-week-old plants were inoculated with over 200 *G. pallida* J2s and left at 19°C (day) / 17°C (night) with 16:8 hours light:dark, with three replicates for each of four time points for each plant species. The plants were washed free of soil at 1, 2, 3 and 4 days for the invasion assays, and at 5, 7, 10 and 14 days for the development assays. The pots of all the replicates of both plant species were positioned randomly. At each time point, the roots were weighed and stained according to the protocol described by Bybd *et al*.(1983), and nematode infection was examined, using a Wild M5 stereo-microscope. Further observations on development were made using one *S. sisymbriifolium* plant at each of 3, 4, 5, 6, 7 and 10 weeks. From 4 weeks onwards, the water residue of the soil from a pot collected in a 250 μm and a 10 μm sieve was examined for the presence of adult females/cysts and adult males, respectively. For the extraction of adult males, the residue collected in a 10 μm sieve was placed on tissue paper on a plastic mesh, which was then immersed in water for 24 h to allow the filtrate to be examined under a microscope. The illustrations by Raski (1950) were used for identification of the developmental stages. Count data (on the log scale) from the invasion assays were analysed by two-way ANOVA to assess the significance of plant species, time and the interaction of these two factors.

Hatching factor

Collection of root exudates

Approximately 50 *S. tuberosum* L. cv. Desirée and *S. sisymbriifolium* plants were grown hydroponically in liquid medium with 16:8 hours light:dark. The temperature was set at 20°C (day) / 18°C (night) for *S. tuberosum* and at 20°C / 20°C for *S. sisymbriifolium*. Sprouts were used for *S. tuberosum*, whereas for *S. sisymbriifolium* seeds were germinated in weed mix. The liquid medium used was adapted from Mulder *et al*. (1996), consisting of Murashige & Skoog (Sigma) with Gamborg's Vitamin solution 1000 × (Sigma) and 0.25 μM IAA (indole-3-acetic acid) (Sigma) at pH 4.2. The medium was changed every two to three days and collected when the plants were 5-weeks-old.

Solid-phase extraction

The liquid medium collected was subjected to gravity filtration (using Whatman No.1 paper) to remove particulate matter, and then to solid-phase extraction (SPE), using a glass C18 SPE column (500 mg, Kinesis). The trapped analytes were extracted with high purity methanol (Rathburn). The extract was dried using dehydrated magnesium sulphate (Fisher Scientific) and the solvent removed using a stream of purified nitrogen until dryness to 0.134 g and 0.03 g for *S. tuberosum* and *S. sisymbriifolium*, respectively.

Electrospray ionisation-mass spectrometry (ESI-MS) analysis for detection of solanoeclepin A

A sample of the residue was prepared for ESI-MS analysis by dissolving a small quantity in high purity methanol (1 mL, Rathburn). An aliquot of the sample (20 μL) was subjected to ESI-MS analysis (Autospec Ultima, Waters, Manchester, UK) (positive ionisation, +4 kV accelerating voltage, mass range = 100–1000 a.m.u.) by injection through a High Performance Liquid Chromatography (HPLC) injection port into a mobile phase (1:1 methanol: water + 1% acetic acid) under the control of an Agilent 1100 LC pump system.

Results

Attraction bioassays

Within 30 minutes of the plantlets being placed in the centre of the Petri dish, approximately 80% of the *G. pallida* J2s were already in the attraction zones of both *Solanum* species, with clear attraction to the roots of *S. sisymbriifolium*. Controls of both species, having no plant material but only the outlines of their corresponding plantlets on the dish lid, contained only *c*. 10%, or less, of the J2s in the attraction zones at any of the time points (Fig. 1).

Statistical analysis showed that there were no significant differences between the two *Solanum* species in attraction of the J2s to their roots ($P = 0.131$). There was also no significance for the plant species-time interaction ($P = 0.328$). However, there was a significant effect of time ($P < 0.001$; the means (natural logs) were: -0.142 (0 min), 2.178 (30 min), 2.325 (60 min) and 2.386 (120 min), SED = 0.1442, 24 df, LSD (5%) = 0.2977 for comparison of these means on the log scale). Most juveniles were attracted within 30 min of exposure in the assay.

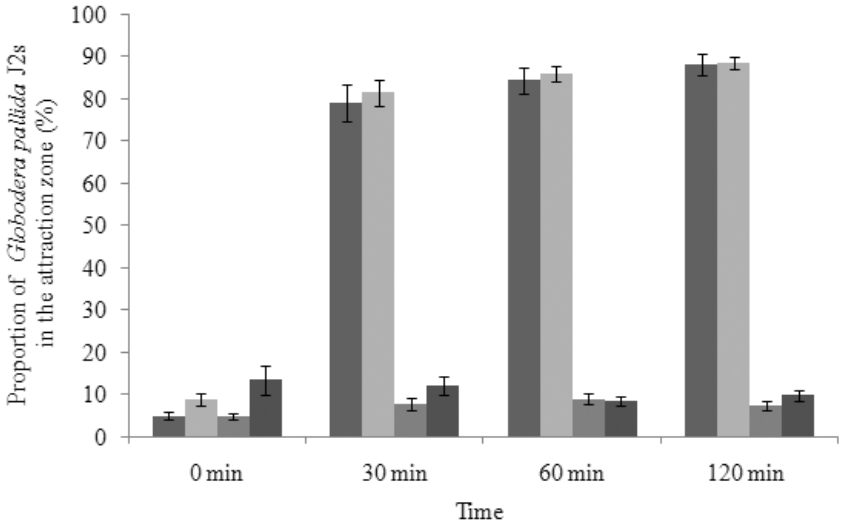

Fig. 1. The mean (n = 5) and standard error of the proportion of *Globodera pallida* J2s that were in the attraction zone at 0, 30, 60 and 120 min after the plantlets were placed in the Petri dishes. Four bars are for (from the left): *Solanum tuberosum* (■), *S. sisymbriifolium* (■), control of *S. tuberosum* (■), control of *S. sisymbriifolium* (■).

Invasion/development assays

Globodera pallida J2s invaded the roots of *S. sisymbriifolium* in large numbers from 1 day post inoculation (dpi). The actual count of the J2s in the roots was much larger in *S. tuberosum* than in *S. sisymbriifolium* at 1 and 4 dpi (data not shown), but when the weight of roots was taken into account, the number of the J2s per gram root of *S. sisymbriifolium* exceeded that of *S. tuberosum* at every time point: 1, 2, 3 and 4 dpi (Fig. 2). The overall difference was significant ($P < 0.001$; the means (natural logs) were 6.923 (*S. sisymbriifolium*) and 5.698 (*S. tuberosum*), SED = 0.1491, 16 df, LSD (5%) = 0.3162 for comparison of these means on the log scale), along with the main effect of time ($P = 0.001$). The interaction was only marginal ($P = 0.047$).

Regarding nematode development, the J2s inside the roots of *S. tuberosum* started to develop at 10 dpi and many third-stage juveniles (J3s), fourth-stage juveniles (J4s) and young females were observed by 14 dpi. However, no development further than J2 was found inside the roots of *S. sisymbriifolium* at 14 dpi, except for one J3 and one slightly more developed J2 in all of the plants examined. In order to see if further development would occur at a later stage, observations were made at 3, 4, 5, 6, 7 and 10 weeks post inoculation (wpi) in one *S. sisymbriifolium* plant at each of these time points. Only J2s were found at 3 and 4 wpi, and at 5 wpi just one moulting J3 was observed with very few other J3s. At 5 wpi, a few J2s were still observed, but at 6 wpi no nematodes were found, with only one nematode (a J3) in the roots at 7 wpi. At 10 wpi, no nematodes were seen in the roots. No adult females/cysts or adult males were found in the soil.

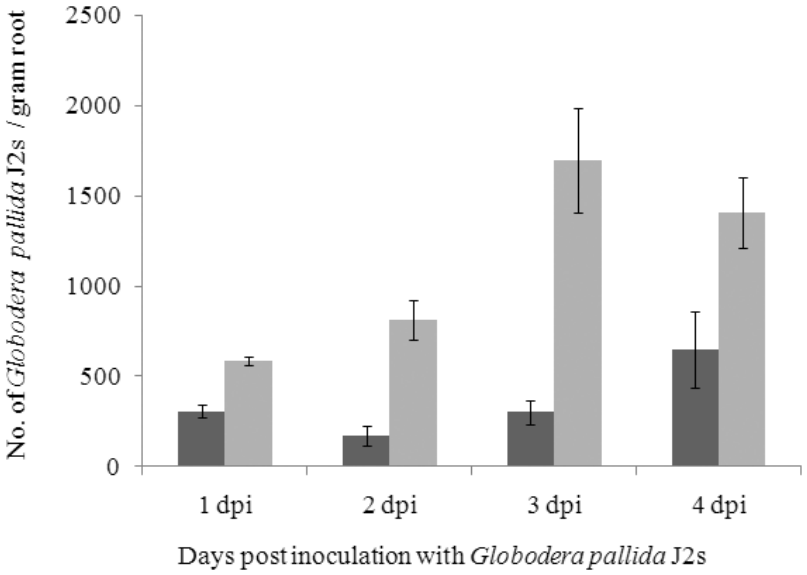

Fig. 2. The mean (n = 3) and standard error of the number of *Globodera pallida* J2s per gram of roots of *Solanum tuberosum* and *S. sisymbriifolium* at 1, 2, 3 and 4 dpi. Two bars are for (from the left): *S. tuberosum* ( ) and *S. sisymbriifolium* ( ).

Hatching factor

ESI-MS analysis of the root exudate extract of *c*. 50 *S. tuberosum* L. cv. Desirée plants revealed the presence of an $[M+H]^+$ peak at m/z 499.9, confirming the presence of the hatching factor, solanoeclepin A ($C_{27}H_{30}O_9$), whose molecular weight is 498.5 (Mulder *et al*., 1996), with the expected $[M+H]^+$ of 499.5 (Fig. 3). However, solanoeclepin A was not detected in the root exudate extract of *S. sisymbriifolium*.

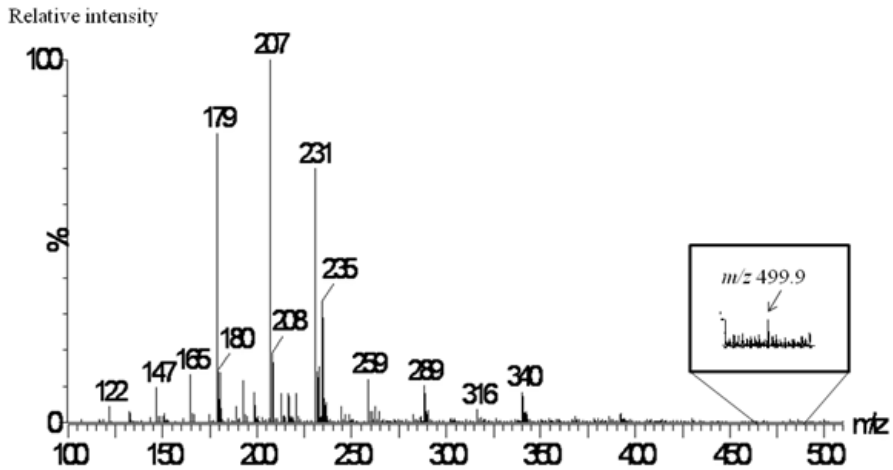

Fig. 3. Mass spectrum of *Solanum tuberosum* L. cv. Desirée root exudate extract revealed an $[M+H]^+$ peak at m/z 499.9, confirming the presence of solanoeclepin A ($C_{27}H_{30}O_9$, molecular weight = 498.5, Mulder *et al*. (1996)).

Discussion

The results of attraction bioassays revealed that *G. pallida* J2s were equally attracted to the roots of *S. sisymbriifolium* and *S. tuberosum* with no statistically significant differences. It was noted that, within 30 mins *c*. 80% of the J2s were already within the attraction zone of both plant species. These results suggest that there are similarities between the two *Solanum* species in the plant attraction signals received by the PCN J2s in their rhizosphere. The signals involved in attracting nematodes can be divided into long-distance (to the root area – a scale of several cm), short-distance (to individual host roots) and local attractants (to preferred invasion sites) (Perry, 2005; Spence *et al*., 2008). From the size of the arena used and the attraction zone defined for these assays, which were 3.5 cm in diameter and 3 mm from the root surface, respectively, it can be suggested that these two plant species have common short-distance attractants to *G. pallida*, rather than long-distance or local attractants.

Interestingly, when it came to invasion, however, significantly more J2s per gram root were observed in *S. sisymbriifolium* than *S. tuberosum*. This may be due to the finer texture of the roots of *S. sisymbriifolium* or more attractive local cues from *S. sisymbriifolium*. And yet those J2s that penetrated the roots of *S. sisymbriifolium* failed to follow the usual development seen in *S. tuberosum*, with no development further than J3. These findings are in line with the report by Roberts & Stone (1983), who found J3s but no J4s, females, cysts or adult males of PCN in the roots of *S. sisymbriifolium* and soil from its pots. The fact that some J2s developed to J3 in the current study, although very few, indicates that a feeding site (syncytium) of sorts was induced. In resistant plants, it is common that although syncytia are initiated by PCN, there is subsequent incompatibility (Hoopes *et al*., 1978; Rice *et al*., 1987; Bleve-Zacheo *et al*., 1990; Sobczak *et al*., 2005). Although it has not been determined, it is highly likely that the limited number of J3s found in *S. sisymbriifolium* were all males, because the fact that nematode development was severely hampered suggests that any induced syncytium was so poor that males, which require much less food than females, would have been produced (Jones & Northcot, 1972).

For a hatching factor, the ESI-MS analysis in this study confirmed that solanoeclepin A was present in the root exudate extract from the hydroponically grown *S. tuberosum* L. cv. Desirée, but not in that from *S. sisymbriifolium*, thus suggesting that *S. sisymbriifolium* roots exude a different hatching factor from *S. tuberosum*.

The overall results so far suggest that the two *Solanum* species exude different hatching factors for *G. pallida*, but send common signals to attract the J2s towards the root area. The J2s invade the roots of *S. sisymbriifolium* and migrate, but their development seems to be prevented by some mechanism. Further investigation is under way.

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The use of Pluronic F-127 to study the development of the potato cyst nematode, *Globodera pallida*

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Summary – This paper demonstrates a simple novel *in vitro* method using Pluronic F-127 aqueous solution to study the development of the potato cyst nematode, *Globodera pallida*, in *Solanum* spp. without any need for sterilisation of either the plants or the nematodes. In this study, this method was successfully applied to comparative studies on the development of *G. pallida* in *Solanum tuberosum* (potato) or *S. sisymbriifolium* (sticky nightshade). The protocol described here could be useful for screening transgenic plants or different plant cultivars/species for their ability to allow development not only of *G. pallida* but also any other plant-parasitic nematodes.

Keywords – nematode development, novel *in vitro* method, *Solanum sisymbriifolium*, *Solanum tuberosum*.

Potato cyst nematodes (PCN), *Globodera pallida* Stone and *G. rostochiensis* (Woll.) Skarbilovich, are economically important pests of potato, *Solanum tuberosum*, in potato-growing regions world-wide (Brodie *et al.*, 1993). Infective second-stage juveniles (J2), after hatching in response to chemical cues from the host plant, penetrate the roots and migrate towards the stele, where they induce multinucleate feeding sites (Jones, 1981; Hussey, 1989; Golinowski *et al.*, 1997). The females of PCN swell and rupture the root after three further moults from J2 to adult. The adult males leave the root after regaining a vermiform shape and fertilise the females. After death, the body wall of the female tans to form a protective cyst that contains several hundred eggs (Perry, 2002; Curtis, 2008).

Study of nematode development in any plant of interest is important to assess the compatibility between the nematode and the plant. This is usually done in pot experiments using soil. However, *in vitro* inoculation of plant roots with infective J2 is more suitable for continuous developmental observations, and usually an axenic medium such as agar is used, but a lengthy process of sterilisation of every material, particularly nematodes, is required.

We describe a simple alternative method where nematode development can be assessed under non-sterile conditions using Pluronic F-127 aqueous solution. Previously, Pluronic gel has been shown to be useful for studying the behaviour of plant-parasitic nematodes (Wang *et al.*, 2009; Sasaki-Crawley *et al.*, 2010; Dutta et al., 2011; Reynolds *et al.*, 2011). An important characteristic of Pluronic F-127 is that it does not form a rigid gel, unlike agar (Gardener & Jones, 1984), and this allows nematodes to move freely in three dimensions (Wang *et al.*, 2009).

Pluronic F-127 is a non-ionic surfactant, polyoxyethylene-polyoxypropylene-polyoxyethylene (PEOPPO-PEO) triblock co-polymer (Bohorquez *et al.*, 1999), and contains only a low level of substrate impurities (Gardener & Jones, 1984). Thanks to this attribute, Ko and Van Gundy (1988) reported that a hatching experiment with *Meloidogyne incognita* was successfully conducted under non-sterile conditions. Here, we demonstrate that Pluronic gel is a suitable matrix for investigating *G. pallida* development in *Solanum* spp. for long periods of time without subjecting the nematode or the seed/plantlet to sterilisation procedures.

Fig. 1. Solanum sisymbriifolium *at 6 weeks post inoculation. Scale in cm.*

Materials and methods

MEDIUM

Twenty percent (w/v) of Pluronic F-127 (Sigma-Aldrich, St Louis, MO, USA) in distilled water including quarter-strength Murashige and Skoog basal salt mixture (Sigma-Aldrich) was placed in a large Duran bottle. The gel was dissolved during sterilisation in an autoclave set at 121°C and kept at 5°C. Plantlets were transferred to Petri dishes containing fresh medium by simply placing them briefly over an ice bath, approximately once a week. This slight decrease in temperature is enough to liquefy the gel allowing the plantlets to be easily lifted without any damage to them.

NEMATODES AND PLANTS

Cysts of *G. pallida* stored at 5°C were first soaked in tap water for 3-5 days to hydrate, and then water was replaced with potato root diffusate (PRD) diluted four times with water and incubated at *ca* 18°C to obtain J2 (Clarke & Perry, 1977).

Solanum tuberosum cv. Désirée plantlets were grown from sprouts by soaking them in water. *Solanum sisymbriifolium* seedlings were grown from seeds (Branston, Lincoln, UK) in a small multi-cell tray with weed mix (80% sterilised loam, 20% lime free grit (3-6 mm), 2 kg m^{-3} osmocote) at 20°C with 16:8 h light:dark.

NEMATODE DEVELOPMENT ASSAYS

In a 3.5 cm diam. Petri dish, *ca* 150 J2 of *G. pallida* in tap water (5-10 μl) and Pluronic gel (*ca* 2 ml) were mixed. A *S. tuberosum* or *S. sisymbriifolium* (10- to 14-day-old) plantlet was placed in the Pluronic medium. The Petri dish was then covered with a small piece of aluminium foil with the aerial part of the plantlet exposed. The plantlet was placed inside a magenta box, with tissue saturated with distilled water laid at the bottom of the box (Fig. 1), which was then incubated at 22°C with 16:8 h light:dark. The plantlets were removed from the medium at 19, 20 and 22 days post inoculation (dpi) for *S. tuberosum* and 19, 20 and 33 dpi for *S. sisymbriifolium*, and the roots were stained with acid fuchsin according to the protocol described by Byrd *et al*. (1983). For *S. tuberosum*, observation was made at 33 dpi, without staining the roots. Photographs were taken to record nematode development using either a Leica M205FA stereo microscope with Leica Application Suite software or an Olympus inverted microscope with Openlab software.

Results

In the roots of *S. tuberosum*, J2 successfully developed to females (Fig. 2A) at every time point (19, 20, 22, 33 dpi), which was repeated in 11 plantlets. Developed females were filled with eggs (Fig. 2B) and adult males were also detected. At 33 dpi an adult male was observed mating with an adult female (Fig. 2C) in two separate plantlets, which suggests that female pheromones were properly dispersed in this medium for an adult male to locate an adult female. It demonstrates that this simple system can facilitate observation of *G. pallida* developmental stages in *S. tuberosum* even until fertilisation.

By contrast, *G. pallida* J2 in *S. sisymbriifolium* showed no further development at every time point in all of the seven plantlets observed even at 33 dpi (Fig. 2D), and only one parasitic J2 was found slightly more developed at 20 dpi, which is in agreement with the data obtained from pot experiments (Sasaki-Crawley *et al.*, 2010).

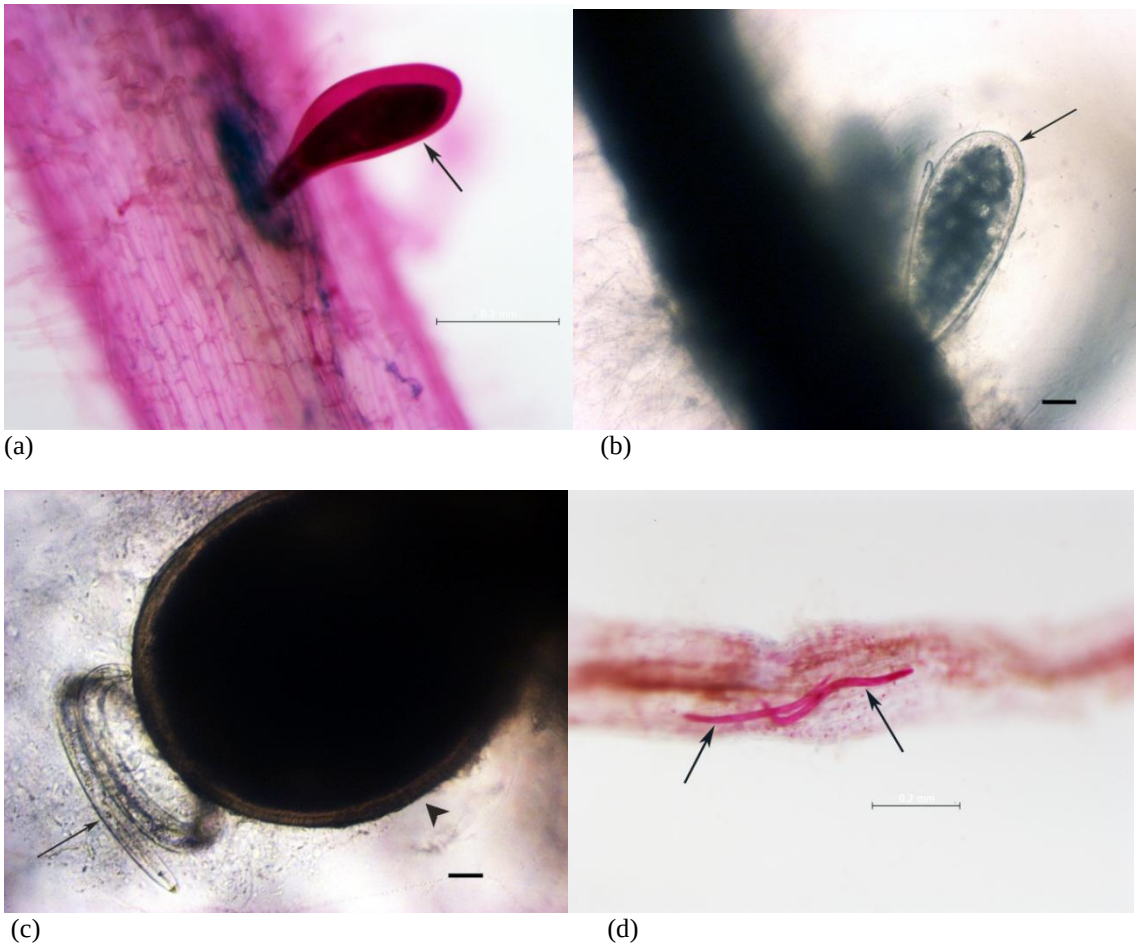

Fig. 2. *A: Female of* Globodera pallida *(arrowed) in the root of* Solanum tuberosum *at 19 dpi stained with acid fuchsin (scale bar = 0.2 mm); B: Female of* G. pallida *(arrowed) filled with eggs in the root of* Solanum tuberosum *at 19 dpi (scale bar = 50 µm); C: Mating between an adult male (arrowed) and an adult female (arrow head) of* G. pallida *in the root of* S. tuberosum *at 33 dpi (scale bar = 50 µm); D: Second-stage juvenile (arrowed) of* G. pallida *in the root of* S. sisymbriifolium *at 33 dpi stained with acid fuchsin (scale bar = 0.2 mm).*

Discussion

The use of Pluronic gel to observe nematode development has notable advantages over axenic cultures using agar plates. Firstly, surface sterilisation of cysts, J2 or plant material is not necessary. Surface sterilisation of infective stages of plant-parasitic nematodes with chemical applications may have an adverse effect on nematodes, such as increased mortality or a possible decline in infecting abilities (Saleh & Fattah, 1990). Antibiotics are often applied, but Forrest *et al*. (1988) reported that cetyltrimethylammonium bromide altered the characteristic structure of the amphidial exudate, thus reducing the ability of *G. rostochiensis* J2 to detect attractants. Heungens *et al*. (1996) described a new rapid method where *ca* 30 *G. pallida* cysts were sterilised with ethanol and sodium hypochlorite in a 20 ml syringe, but the process becomes much more complicated when a large number of cysts (a scale of several hundred) have to be sterilised.

Secondly, due to its non-rigid texture and transparency, Pluronic gel is an ideal medium for nematode movement in three dimensions, and the infection process is easily observed under a microscope. Also, all roots can be subjected to nematode invasion as opposed to only those exposed on the surface of the agar plates. This affords the potential for more realistic evaluations of nematode-host interactions.

Thirdly, Pluronic gel liquefies when the temperature falls below a certain level for a certain concentration (Gardener & Jones, 1984), which allows the infection process to be easily synchronised. This can be done simply by lowering the temperature of the Pluronic gel and then placing the whole plant into another container with fresh Pluronic gel without nematodes. This process is harmless to the plant and allows a crude estimation of the number of J2 that have penetrated the roots by counting the number left behind in the original container. Since the medium can be changed as required, the growth of plants can be sustained for unlimited time. Figure 1 shows an example with *S. sisymbriifolium*, where the health of the seedling is clearly seen at 6 weeks post inoculation.

The protocol described here has allowed observation of the life-cycle of *G. pallida* in *S. tuberosum*, and comparative studies were successfully conducted without subjecting the nematode or the seed/plantlet to sterilisation procedures. It could be very useful for screening transgenic or resistant plants or various plant cultivars for their ability to allow the development of plant-parasitic nematodes, and also offers a very useful platform for the collection of different nematode parasitic stages for sequencing or proteomics analysis. The application of this protocol can be extended to the *in vitro* study of the negative/positive interactions of herbivore attack above (insect) and below-ground (nematode) on each other through their shared host plant (Wurst & van der Putten, 2007; Kaplan *et al.*, 2008; Hong *et al.*, 2010) or the effect of above- and below-ground herbivory on the host plant (Kaplan *et al.*, 2008).

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